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Screening of Yeasts Isolated from Agro-Material for Alcohol Fermentation Potential

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A B S T R A C T

Yeasts are the most important microorganisms during alcoholic fermentation because they influence fermentation speed, wine flavour and other wine qualities. The aim of this study was to Screen Yeasts Isolated from Agro-Material for Alcohol Fermentation Potential. Agro-materials used were palm wine and pineapple waste. The palm wine was left to ferment for five days. Both the fermented palm wine and the juice extracted were serially diluted in peptone water. From the diluted palm-wine and pineapple juice sample, 0.1ml of 10^{-5} and 10^{-6} dilutions were inoculated onto the PDA plates and spread-plated. The inoculated plates were incubated at 30°C for 48 – 72 hours, after which colonies were primarily identified by morphological and microscopic methods. Further identification was done using molecular techniques. TheIdentified isolates were screened for their tolerance ability. Yeast isolates were screened for Alcohol tolerance (0% to 15%), pH tolerance (pH 2 to pH 4) and sugar tolerance (0% to 20%). The results of the morphological characterization of the yeast showed that Isolates Pn3, Pw7 and Pw9 matched the description of *Candida specie*, isolate Pn4 identified as *Pichia species*, while isolates Pn5, Pw4, Pw5, Pw6 and Pw11 were identified as *Saccharomyces cerevisiae*. Results from molecular characterisation of the selected yeast isolates shows that Isolate PwP11 identifies as *Saccharomyces cerevisiae* strain YBA 08 which has NCBI accession number MN158119.1, PnY4 as *Pichia kudriavzevii* isolate A60G10 which has NCBI accession number MF285861.1 and PwP9 as *Candida nivariensis* strain CNRMA6.84 which has NCBI accession number KP131741.1. The yeast isolate for fermentation of ethanol screened revealed that *Saccharomyces cerevisiae* strain YBA 08 from 3 days old Palm wine and *Pichia specie* from 3days old Pineapple juice has the highest reading for pH tolerance, ethanol tolerance and sugar tolerance. These results reveal that both isolates had the ability to breakdown sugar containing substrate to alcohol and can tolerate pH environment.

Introduction

Yeasts are single-celled microorganisms that are classified, along with molds and mushrooms, as members of the Kingdom Fungi. Their vegetative growth result predominantly from budding or fission.

Yeast aids in the fermentation of sugars (like disaccharides; sucrose, lactose, and monosaccharides; fructose, glucose) to produce ethanol in an anaerobic pathway called glycolysis, where the monosaccharide are metabolised into two molecules of pyruvate and the pyruvate is further reduced to ethanol and carbon-dioxide by the enzymes pyruvate decarboxylase and alcohol dehydrogenase. The reaction is presented as;

$$\underset{\text{(Glucose)}}{C_6H_{12}O_6} \longrightarrow \underset{\text{(Ethanol)}}{2C_2H_5OH} + \underset{\text{(Carbon-dioxide)}}{2CO_2}$$

Yeasts are the most important microorganisms during alcoholic fermentation, as they influence fermentation speed, wine flavour and other wine qualities (Pretorius *et al.,* 2006). Fleet *et al.,* (2003) explained that the *Saccharomyces* genus was most commonly used in beverage industry due to their higher capacity to ferment sugars which allowed them to colonise sugar-rich media over other yeasts, which were not tolerant to sugar media.

Saccharomyces cerevisiae has been confirmed as the dominant yeast species responsible for the fermentation of palm wine tapped from the felled palm trees (Amoa-Awua *et al.,* 2007).Alcohol fermentation process is usually carried out by species of the yeast *Saccharomyces*, whereby the sugars in the substrate are converted to alcohol and organic acid, that later react to form aldehydes, esters and other chemical components (Isitua and Ibeh, 2010).Joshi *et al.,* (2009), stated that for alcohol production, the strains commonly used belonged to the species *Saccharomyces cerevisiae* or *Saccharomyces bayanus* and the choice of yeast strain as starting culture could have a high impact on the flavour profile of fermented beverages.

Saccharomyces cerevisiae and *Zymomonas mobilis* are capable of producing about 8% ethanol from pineapple waste in 48 h after pretreating with the enzymes cellulase and hemi-cellulase (Upadhyay *et al.,* 2010). However, fermentable sugars which include sucrose, glucose and fructose are reported to be relatively low, thus pre-treatment of the substrate with enzymes like cellulase and hemi-cellulase are necessary for alcohol production.

Yeast species such as *Kluyveromyces marxianus*, few *Sacchromyces cerevisiae* and *Pichia kudriavzevii* are capable of producing ethanol at temperature range of 40 to 45 °C (Chamnipa *et al.,* 2018; Limtong *et al.,* 2007; Nuanpeng *et al.,* 2016; Nwuche *et al.,* 2018). *P. kudriavzevii* is exceptionally stress tolerant and has a growing role in bioethanol production (Mukherjee *et al.,* 2017) and several *P. kudriavzevii* strains have been reported to grow and produce ethanol effectively at high temperatures (Chamnipa *et al.,* 2018; Dhaliwal *et al.,* 2011; Oberoi *et al.,* 2012; Yuangsaard *et al.,* 2013). However, only a few strains of *P. kudriavzevii* (Isono *et al.,* 2012; Kwon *et al.,* 2011) have been studied for ethanol production under multiple stress conditions.

Ali and Khan (2014) stated that yeast strains associated with fruit surfaces can convert a wide range of sugars into alcohol, thereby they can tolerate high alcohol concentrations and that specific physiological properties (like ethanol tolerance and sugar tolerance) are required when evaluating a yeast strain for use in industrial ethanol production.

Materials and Methods

Freshly tapped Palm wine sap was collected using a sterile container while wholesome pineapple fruit was purchased within Port-Harcourt metropolis, Rivers State and transported in a sterile polythene bag to microbiology laboratory of the Department of Microbiology, Rivers State University. The pineapple fruit was washed with water containing metabisulphide (2%), cut and the pineapple waste (core and peel) was blended using a sterile blender. The juice was extracted and kept in a sterile flask.

Isolation and subculture of yeast isolates

The palm wine sap was left to ferment for five days. Using the method used by Odu *et al.,* (2020), serial dilution was performed on the fermented palm wine sap, 1ml of the liquid was serially diluted with peptone water in a tenfold serial dilution, after which an aliquot (0.1ml) of 10^{-5} and 10^{-6} dilutions were inoculated onto the Potato Dextrose Agar plates and spread-plated. Potato Dextrose Agar (PDA) was prepared according to manufacturer's guide and sterilised by autoclaving at 121^{o}C for 15mins at 15 PSI.

From the pineapple waste (core and peel) juice extracted after blending, 1ml was serially diluted with peptone water in a tenfold serial dilution. From the sample, 0.1ml of 10^{-5} and 10^{-6} dilutions were inoculated onto the PDA plates and spread-plated using a sterile bent glass rod. The inoculated plates were incubated at 30°C for 48 – 72 hours, after which colonies were identified for unique characteristics and purified by sub-culturing on sterile PDA plates. The obtained pure cultures were characterized, identified, and screened.

The morphology of the yeast was identified through macroscopic (appearance of colony on PDA plates like colour, shape and texture) and microscopically by viewing under the light microscope at ×100 magnifications after staining (Nnodim *et al.,* 2021)

The yeast isolates were further identified molecularly (PCR and sequencing).

Molecular Identification of Selected Yeast Isolate

The procedure was carried out at the laboratory of Bioinformatics Service Laboratory, Mokola Ibadan, Oyo State, Nigeria.

The DNA extraction was done using ZR fungal/bacterial DNA mini prep (manufactured by zymo research). Two (2) ml of cell broth was added to a ZR Bashing^{TM}Lysis Tube and 750 microlitre (ul) Lysis Solution was added to the tube. The tubes were secure in a bead fitted with 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR Bashing Bead^{TM} Lysis Tube was centrifuged in a micro-centrifuge at 10,000 rpm for 1 minute. Four hundred (400) µl supernatant was transferred to a Zymo-Spin^{TM} IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 rpm for 1 minute. One thousand two hundred (1,200) µl of Fungal/Bacterial DNA Binding Buffer was added to the filtrate in the Collection Tube. After which, 800 µl of the mixture was transferred from the remaining volume in the collection tube to a Zymo-Spin^{TM} IIC Column in a Collection Tube and centrifuged at 10,000 rpm for 1 minute. The flow was discarded through the Collection Tube and Step 6 was repeated. Two hundred (200) µl of DNA Pre-Wash Buffer was added to the Zymo-Spin^{TM} IIC Column in a new Collection Tube and centrifuged at 10,000 rpm for 1 minute. Five hundred (500) µl Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin^{TM} IIC Column and centrifuged at 10,000 rpm for 1 minute. The Zymo-Spin^{TM} IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100ul (35 ul minimum) DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 rpm for 30 seconds to elute the DNA (Wilfinger *et al.,* 1997)

The PCR mix is made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270). 1µL each of 10µM forward and reverse primer; 2µL of DNA template and then made up with 8.5µL Nuclease free water.

Primer sequences for yeast

ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'

ITS4 5' TCCTCCGCTTATTGACATGS 3'

Agarose Gel Electrophoresis of Amplified DNA and PCR Molecules

One gram (1g) of agarose (for DNA) was mixed and 2g of agarose for PCR was mixed with 100 mL 1xTAE in a microwavable flask. The mixture was

put in the microwave for 1-3 min until the agarose was completely dissolved (caution was taken as to not over boil the solution, cause some of the buffer will evaporate and thus alter the final percentage of agarose in the gel).

The agarose solution was kept to cool down to about 50°C for about 5 mins.10µL of EZ vision DNA stain was added (EZ vision binds to the DNA and allows for the visualization of the DNA under ultraviolet (UV) light).The agarose was poured into a gel tray with the well comb in place. The poured gel was let to sit at room temperature for 20-30 minutes, until it has completely solidified.

Loading buffer was added to each of the extracted DNA samples. Once solidified, the agarose gel was placed into the gel box (electrophoresis unit).The gel box was filled with 1xTAE (or TBE) until the gel was covered. A molecular weight ladder was loaded into the first lane of the gel and the samples were carefully loaded into the additional wells of the gel. The gel was subjected to run at 80-150 V for about 1-1.5 hours, after which the power was turned off and the electrodes was disconnected from the power source and then the gel was carefully removed from the gel box. DNA fragments or PCR product was visualized under UV trans-illuminator (Nnodim *et al.*, 2021).

Cycling Conditions

Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45se, followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10 ˚C.

Sequencing of Amplified Isolates

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA X were used for all genetic analysis.

Evolutionary Relationships of Taxa

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length was taken to represent the evolutionary history of the taxa analysed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. This analysis involved 3 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). For bacteria, evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). While for yeast, there were a total of 827 positions in the final dataset and the evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).

Screening of Isolated Yeast for Alcohol Production Potential

Identified isolates were screened for their tolerance ability. Yeast isolates were screened for Alcohol tolerance (0% to 15%), pH tolerance (pH 2 to pH 4) and sugar tolerance (0% to 20%).

Ethanol tolerance

The identified Isolates were screened for their ability to withstand several ethanol concentrations and their ability to produce in such concentrations. Peptone water was prepared by suspending 15g in 1000ml of distilled water.

Then concentration of ethanol of 5%, 10%, and 15% differing by 5% v/v were prepared from one flask to another in ratio of 95:5, 90:10, and 85:15 respectively against the medium containing 0% ethanol as control. 9ml of the mixture was dispensed in 10ml test tubes and was autoclaved at 121^{o}C for 15mins at 15psi. Using a sterilised wireloop, a

loopful of the selected yeast isolates were inoculated in the test tubes and the inoculated test tubes were incubated at 37^{o}C for 24hour. After incubation, microbial growth was determined via turbidity measurement using a light spectrophotometer set at a wavelength of 600 nm, the initial and final optical density was recorded (Alabere *et al.*, 2020; Ali and Khan, 2014). All experiments were carried out in duplicates and mean values were considered

pH tolerance

Isolates were screened for their ability to tolerate high acidic environment. Isolates were subjected to grow in pH 2, 2.5, 3, 3.5 and 4 levels (in duplicates). Peptone water was prepared by suspending 15g of peptone in 1000ml of distilled water and pH was adjusted using 4M of sodium hydroxide (NaOH) and 4M of hydrochloric acid HCL. 9ml of the mixture was dispensed in 10ml test tubes and was autoclaved at 121^{o}C for 15mins at 15psi and a loopful of the yeast isolates were inoculated.

The turbidity was measured by spectrophotometer at 600 nm after inoculation and incubated at 37^{0}C for 24hours. After incubation, microbial growth was determined via turbidity measurement using a light spectrophotometer set at a wavelength of 600 nm (Alabere *et al.*, 2020).

Sugar tolerance

Sugar (sucrose) concentrations 5%, 10%, 15% and 20% were prepared using peptone water as a medium. Nine millimetres of the prepared concentrations were dispensed in 10ml test tubes in duplicates and was autoclaved at 121^{o}C for 15mins at 15psi**.**

A loopful of the isolate was inoculated in the test tubes and the turbidity was measured by spectrophotometer at 600 nm after inoculation. The test tubes were incubated at 37^{o}C for 24hours and after incubation, the optical density readings were taken using a spectrophotometer set at a wavelength of 600 nm.

Statistical analysis

All experiments were calculated using the software MINITAB. A two-way analysis of variance (ANOVA) and mean separation using tukey form was carried out. The data was presented in graphic form.

Results and Discussion

Table 1 shows the morphological characterization of the yeast isolates and isolates Pn3, Pw7 and Pw9 matched the description of *Candida species*, isolate Pn4 identified as *Pichia species*, while isolates Pn5, Pw4, Pw5, Pw6 and Pw11 were identified as *Saccharomyces cerevisiae*.

The sequence identification from NCBI BLAST hits and their percentage relatedness is shown in Table 2.Isolate PwP11 identifies as *Saccharomyces cerevisiae* strain YBA 08 which has NCBI accession number MN158119.1 and the percentage relatedness was 91.69%, PnY4 as *Pichia kudriavzevii* isolate A60G10 which has NCBI accession number MF285861.1 and the percentage relatedness was 91.75%, and PwP9 as *Candida nivariensis* strain CNRMA6.84 which has NCBI accession number KP131741.1 and the percentage relatedness was 92.24%. Shown in Fig. 1 is the agarose electrophoresis of amplified 18S rRNA gene of the yeast isolates which shows that the ITS region of the isolates were at about 550bp, while the evolutionary relationship of the yeast isolates is shown in Fig. 2.

Screening of Yeasts for Their Tolerance to Ethanol, pH and Sugar

The yeast isolate for fermentation of ethanol were screened to find out their survival level and the results indicates that *Saccharomyces cerevisiae* from 3days old Palm wine and *Pichia kudriavzevii* also from 3days old Pineapple juice has the highest reading for pH tolerance, ethanol tolerance and sugar tolerance. This result reveals that both isolates had the ability to breakdown sugar containing substrate to alcohol and can tolerate pH levels of 2

to 4, ethanol concentration of 5 to 15% and sugar concentration of 5 to 20%. The factorial plots from the results are shown in figures 3 – 5. The plots for absorbance versus alcohol concentration*isolate is shown in Figure 3, *Saccharomyces cerevisiae* had a better progression from 0% to 15% unlike that of *Candida nivarensis* that survived at 0% to 5% and dropped at 10%. Figure 4 shows the plots for absorbance versus sucrose concentration*isolate, this graph indicated that *Saccharomyces cerevisiae* had the best tolerance rate and could grow and tolerate sugar concentrations up to 20%, *Candida nivarensis* could not tolerate sugar environment of 20% and *Pichia kudriavzevii* struggled to survive in a sugar concentrated environment. The plots for absorbance versus pH value*isolate is shown in Figure 5, it shows that *Pichia kudriavzevii* can survive in acidic environment and it also indicates that *Saccharomyces cerevisiae* can survive best in an environment of pH value of 3.

Yeasts are widely distributed in nature thus naturally found in environments like fruits (Singh and Sharma, 2007). The macroscopic and microscopic morphological characteristics of the yeast isolates are shown in Table 4.1. The yeast species identified were; *Saccharomyces sp.* and *Candida sp*. *Saccharomyces sp.* and *Candida sp.* were isolated from Palm-wine, another isolate of *Saccharomyces sp.* was isolated from Pineapple juice and this finding agrees with Nwakanma *et al.,* (2015) that isolated *Saccharomyces cerevisiae* from palm-wine gotten from different sites. This indicates that *Saccharomyces cerevisiae* is a natural microbial flora in palm-wine. Our results show that most of the isolates were *Saccharomyces cerevisiae* gotten from the 5day old Palm-wine used for isolation.

The obtained 18S rRNA and a 16S rDNA sequence from the isolate produced during megablast search an exact match of similar sequences from the NCBI non-reductant nucleotide (nr/nt) database. The 18S rRNA and 16S rDNA of the isolate showed percentages of 80 – 100% similarity to other species. BLAST analysis of the obtained sequences for yeast isolates showed 91.69%, 91.75% and 92.24% sequence identity with *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Candida nivariensis* for isolates Pw11, Pn4 and Pw9 respectively as shown in table 2.

Figure 3 - 5 suggests that the isolates screened were tolerant to ethanol, sugar and pH factors but some isolates like *Saccharomyces cerevisiae* showed stronger ability to the factors. Sugar concentration is an important critical factor in fermentation process and it influences the rate of production and the yield in addition to physiological growth of yeast.

Alcohol Tolerance

Figure 3 shows, isolates screened for their tolerance to various concentrations of alcohol (5%, 10%, and 15% against 0% as control), *Pichia kudriavzevii* survived best in all concentrations, while *Saccharomyces cerevisiae* survived best in alcohol concentration of 15%. This agrees with the study by Kumar *et al.,* (2011), the authors tested the effect of ethanol concentration on the cell growth and viability of *Saccharomyces cerevisiae* and concluded that *Saccharomyces cerevisiae* tolerate up to 15% of ethanol in the medium.

Similar results were reported by Khaing *et al.,* (2008). They reported that the *S. cerevisiae* (KY1&KY3) strains has tolerance up to 15% of ethanol in the medium and *S. cerevisiae* (KY2) tolerate up to 20% of ethanol leading to maximum ethanol production over a long incubation period.

Sugar Tolerance

The yeast isolates were also subjected to 4levels of sugar concentrations (5%, 10%, 15%, 20% and 0% as control) and fig. 4 showed that *Saccharomyces cerevisiae* survived best in all concentrations of sugar especially at 20% concentration and this agrees with the findings by Ali and Khan (2014) that stated among all strains in their study, *S. cerevisiae* expressed highest sugar tolerance of upto 20%.

Table.1 Macroscopic and Microscopic Characteristics of Yeast Isolates

Isolates	Macroscopic	Microscopy	Probable organism
Pn3	Smooth surface, cream colour, raised	Ellipsoidal, budding blastoconidia, absence of pseudohyphae	*Candida sp.*
Pn4	Smooth, cream colour to yellowish, rapid growth.	Predominantly small, elongated to ovoid blastoconidia	*Pichia sp.*
Pn5	Raised, rough edges, dry surface and cream color	Ovoid blastopores	*Saccharomyces sp.*
Pw4	Whitish in colour, smooth surface, dry and raised	Spherical Ovoid cells in clusters	*Saccharomyces cerevisiae*
Pw5	Flat, smooth surface, moist and creamy in color	Spherical Ovoid cells in clusters	*Saccharomyces sp.*
Pw6	Cream color, slightly raised, smooth surface	Spherical Ovoid cells in clusters	*Saccharomyces sp.*
Pw7	Light cream color, smooth surface, raised and had an entire margin	Ellipsoidal, budding blastoconidia, absence of pseudohyphae	*Candida sp.*
Pw9	Raised, Cream in colour and small round colony	Ellipsoidal, budding blastoconidia, absence of pseudohyphae	*Candida sp.*
Pw11	raised, circular, smooth surface, moist and creamy in color	Spherical Ovoid cells in clusters	*Saccharomyces sp.*

Key: Pn = Pineapple sample, Pw = Palm-wine sample

Table.2 Sequence identification from National Centre for Biotechnology Information (NCBI) BLAST Hits and their Percentage Relatedness for Yeast Isolate

S/N	Sequence code	NCBI BLAST relative	Accession number	E value	% relatedness
1	Pw11	*Saccharomyces cerevisiae* YBA 08 AVP1	MN158119.1	0	91.69
2	Pn4	*Pichiakudriavzevii* A60G10	MF285861.1	6e-168	91.75
3	Pw9	*Candida nivariensis* CNRMA6.84	KP131741.1	0	92.24

Key: Pw = Palm wine, Pn = Pineapple

Fig.1 ITS PCR Gel Image of yeast isolates

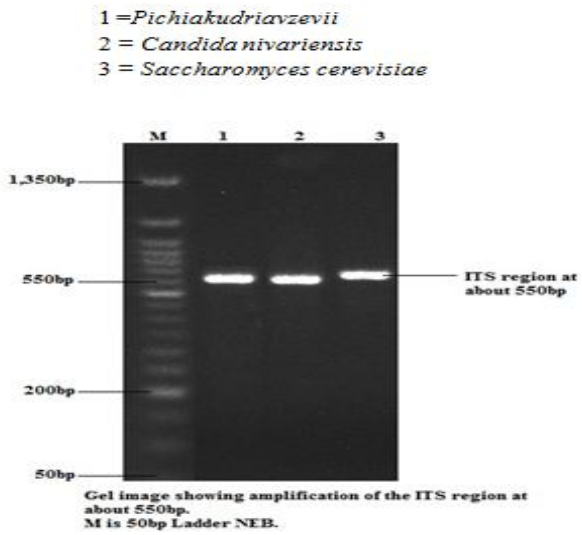


Fig.2 Phylogenetic Tree showing the evolutionary distance between Yeasts Isolates

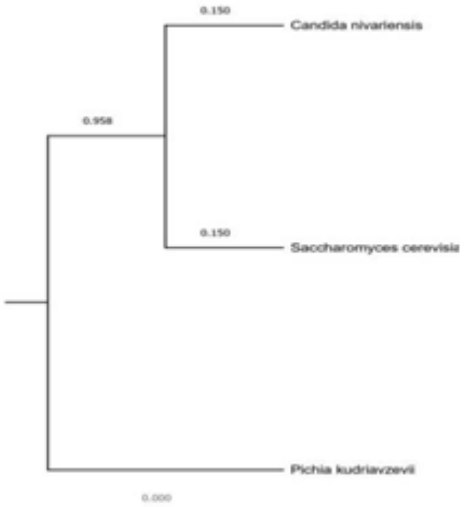


Fig.3 Factorial Plots for Absorbance (OD600nm) versus Alcohol concentration (%)*Isolate

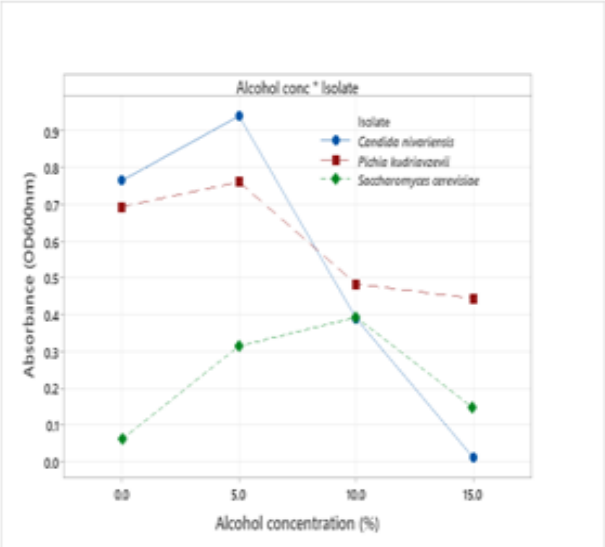


Fig.4 Factorial Plots for Absorbance (OD600nm) versus Alcohol concentration (%)*Isolate

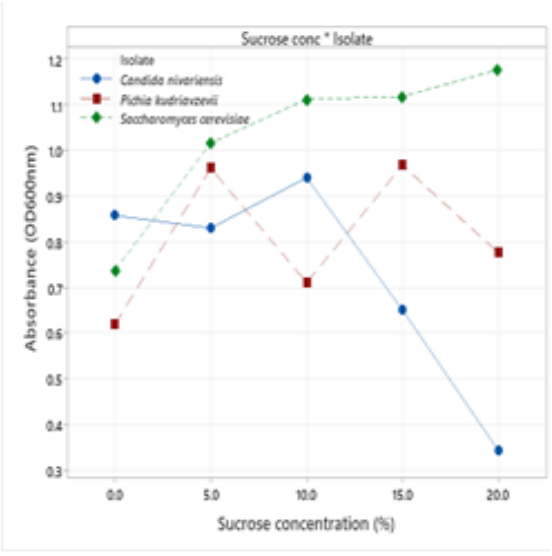
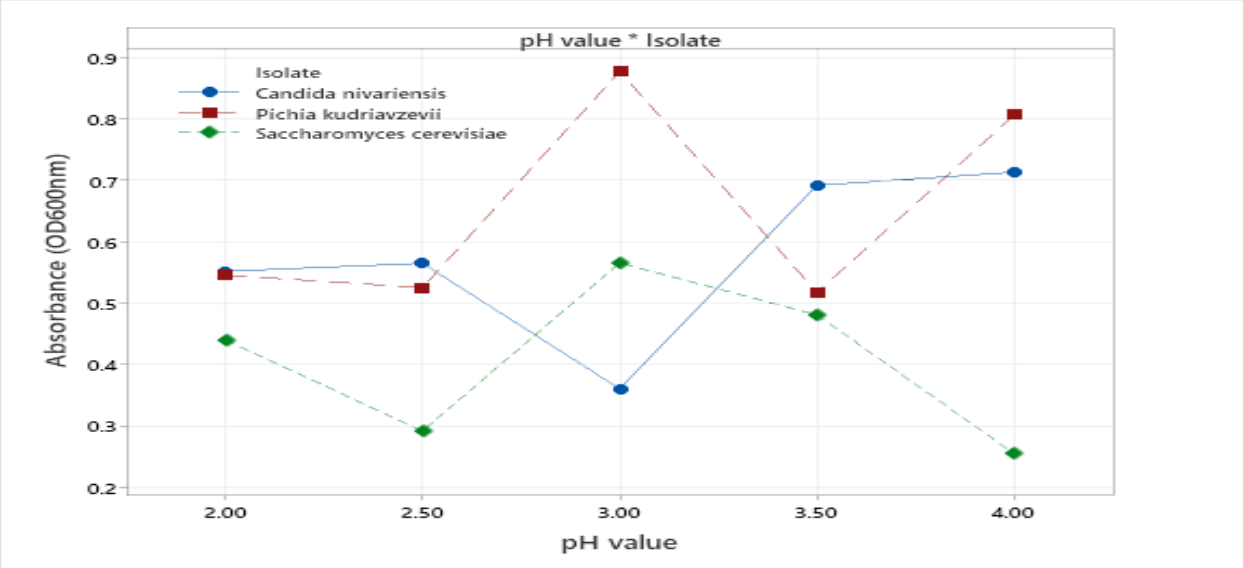


Fig.5 Factorial Plots for Absorbance (OD600nm) versus pH Value*Isolate



This study also correlates with the data obtained for sugar tolerance of wine yeasts by Osho, (2005) who also reported maximum of 20% sugar tolerance for *S. cerevisiae* BSOSU 0269. This study shows that *Candida nivariensis* survived best at 10% sugar concentration, and *Pichia kudriavzevii* survived best at 5 and 15% sugar concentrations.

pH Tolerance

The identified yeasts were also subjected to five levels of pH (2, 2.5, 3, 3.5 and 4) values as shown in fig 4.5.*Pichiakudriavzevii* had the best tolerance to pH value 3, *Candida nivariensis* had the best tolerance at pH 4, while *Saccharomyces cerevisiae* survived best at pH level 3. This supports the study by Manikandan *et al.*, (2010) that *S. cerevisiae* yeast isolated from toddy survived at an optimum pH of 3.0 and initial sugar concentration 20%.

Based on the data obtained for the characterization of isolates, *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Candida nivariensis* were identified from the Agro-materials used as samples. From the tolerance testing carried out, from the three yeasts (*Saccharomyces cerevisiae, Pichia kudriavzevii* and *Candida nivariensis*) screened, *Saccharomyces cerevisiae* had the ability to withstand alcohol concentration of up to 10%, sugar concentration of up to 20%, and pH value of 3 and can be used by industries in the future to produce alcohol.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript

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