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Original Article

Screening for Potential Alcohol-Tolerance Yeasts from Indigenous Substrates as Alternative to Saccharomyces Cerevisae

Ikechukwu Jude Onyia[1], Uzoamaka Ogechi George-Okafor[2], Ujunwa Felicia Nwachukwu[3]

[1,2,3]*Enugu State University of Science and Technology Agbani, Enugu Nigeria, Faculty of Biological Sciences, Department of Applied Microbiology and Brewing.*

[3]*Corresponding Author : nwachukwuujunwa77@yahoo.com*

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Abstract *- Yeasts have been reported to be associated with the production of important products, including ethanol. They can be isolated from many sources, including palm wine and fruit juice. Therefore, this study aimed at isolating and screening for potential alcohol-tolerance yeasts from indigenous substrates as an alternative to Saccharomyces cerevisae. The samples which included fresh palm wine, ripped oranges, carrots, banana and pineapple, were respectively inoculated into potato dextrose agar (PDA) and incubated at 30^{o}C for 48h. Thereafter, the developed colonies were identified through conventional mycological analysis and ITS molecular sequencing. Each identified yeast 1ml (equivalent to 1.52 X 10^{2} CFU/mL) was exposed to different concentrations (6-16%) of absolute ethanol incorporated into 100ml of PDA, respectively. The residual growth was measured spectrophotometrically at 660nm after incubating at 30^{O}C for 48h. The same treatment subjected to commercial S. cerevisae served as control. The recovered isolates included 5 strains of Meyerozyma guilliermondii (19, KAS-143, GTi7, ANSe-23, RCPF 1373), 3 strains of Candida tropicalis strain (L6, OBY6 and IDR1000028827) and Kodamaea ohmeri strain TS21. All the yeast isolates and the control Saccharomyces cerevisae tolerated ethanol even at 16% concentration, but the tolerance rate was significantly higher with Saccharomyces cerevisae across the concentration($p<0.05$). The rate of ethanol tolerance decreased as the ethanol concentration increased in all the yeast isolates, even with the control. Among the isolates, Candida tropicalis strain IDR1000028827 exhibited the highest tolerance rate (80%) at 16% ethanol concentration, while Meyerozyma guilliermondii strains KAS-143 demonstrated the least tolerance (20%) at 16% ethanol concentration). The result obtained from this study revealed the presence of some high ethanol tolerance yeasts, which could be tried for indigenous ethanol production, despite the observed significant difference that existed among them and the commercial yeast.*

Keywords *- Ethanol Tolerance, Indigenous substrates, Isolation, Screening, Yeast.*

1. Introduction

Yeasts are known as unicellular eukaryotic microorganisms, which exist either as free-living or symbionts of plants and animals. They can be utilized in the production of vitamins, ethanol, antibiotics, alcoholic beverages, bread, kefir and other fermented food products. Yeasts can be gotten from many sources, including palm wine and fruit juice. Palm wine is a traditional alcoholic beverage that is appreciated by society and contains nutritional value which helps improve the diet of consumers (Aka et al. 2008; Frances et al. 2023). Its fermentation is mostly carried out by yeast strains, including Saccharomyces cerevisiae strains, which are the dominant strains (Olowonibi 2017). Saccharomyces cerevisiae strains have fermentative and oxidative potentials as they are known to ferment sugars to produce alcohol, mainly ethanol and also carbon dioxide under anaerobic conditions. Several yeast strains including Candida species such as C. tropicalis, C. inconspicua, C. rugosa, C. parapsilosis; Kluyveromyces ma guilliermondii, Rhodotorula mucilaginosa, rxianus, Hanseniaspora guilliermondii, Rhodotorula mucilaginosa, Yarrowia lipolytica, Kodamaea ohmeri and Trichosporon asahii have been isolated from palm wine (Attchelouwa et al. 2018; Egue et al. 2018a; Olowonibi 2017). Apart from palm wine, other substrates have been indicated as sources for yeast isolation (Egue et al., 2022). The most common yeast strains identified from these fruit juices include Saccharomyces cerevisiae, Candida guillermondii, C. krusei, C. famata, C. spherica, C. colliculosa, C. albicans, Trichosporon mucoides, Kloeckera spp. and yeast-like fungus Cryptococcus neoformans. C. guillermondii (Uhiti et al. 2009). Among these yeasts, S. cerevisiae has played a major role in many industries, especially in ethanol production. The continuous utilization of S. cerevisiae in ethanol production is based on its characteristics, including high tolerance to ethanol. The

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organism developed many strategies to overcome ethanol stress by activating the stress response pathway, which regulates many genes involved in modifying the cell membrane cell wall, renaturation of proteins, and altering the metabolism (Varize et al. 2022).

Ethanol tolerance is a very important property for consideration under industrial ethanol production. At higher ethanol concentrations, the yeast cells, including Saccharomyces cerevisiae, may be unable to tolerate ethanol as the ethanol can interact with the yeast cell membrane and disrupt its cellular activities, leading to the death of the cell. Therefore, it is very necessary to isolate these potential yeast strains that can serve as alternatives to commercial S. cerevisiae from indigenous substrates and to subject them to ethanol tolerance tests to identify their tolerance rate in order to ascertain the ethanol concentration in which the yeast cell can survive. The possible recovery of indigenous yeast with equivalent high ethanol tolerance potential with S. cerevisiae can help to reduce production costs resulting from the high cost of S. cerevisiae importation to the country.

2. Materials and Methods

2.1. Collection and Preparation of Samples for Yeast Isolation

The samples utilized in this study were palm wine and fruits, including ripped oranges, carrots, bananas and pineapple. The freshly taped palm wine was purchased from a palm wine taper, and the fruits were purchased from a fruiterer at Eke Agbani local market in Enugu State, Nigeria. All the samples were taken to the microbiology laboratory of Enugu State University of Science and Technology, Nigeria, for assessment. All the fruits (including ripped oranges, carrots, bananas and pineapple) were washed with sterile water and peeled. The carrots and banana were first sprinkled with about 5-10ml of sterile water and ground to homogenous liquor using the home electrical blender. The orange and pineapple were pressed using a citrus extractor (Century Hisense) to extract their juice. Thereafter, the samples were respectively inoculated into Potato Dextrose agar (PDA), and incubated at 30^{o}C for 48h to recover the developed colonies. Further subcultures with PDA resulted in the recovery of pure cultures for identification.

2.2. Identification of the Isolates

The predominant isolates were first observed for colonial appearances on potato dextrose agar culture prior to staining with lactophenol cotton blue and Gram reagents or morphological identification through microscopic examination using X40 and X100 objective lenses, respectively. Only the probable yeast isolates were subjected to molecular characterization, as stated below. The DNA of each isolate was first extracted using ZR fungal/bacterial DNA MINIPREP (Manufactured by Zymo Research), and its purity and amplification through electrophoresis and PCR were carried out as described by Nwachukwu et al. (2023). Their internal Transcribed Spacer (ITS) gene sequencing and phylogenetic analysis were carried out as described by George-Okafor *et al.* (2022). Each identified yeast was then subjected to ethanol tolerance capabilities.

2.3. Ethanol Tolerance Assay

Each yeast isolate (1% w/v) representing (1.52 X10^2 CFU/mL) cell was seeded into PDA broth containing ethanol in the concentration of 6, 8, 10, 12, 14 and 16% respectively and incubated at 30^{o}C for 48h. The growth was measured using a spectrophotometer at 520nm. The culture containing commercial *Saccharomyces cerevisiae* served as control.

2.4. Determination of the Percentage Ethanol Tolerance Assay

The percentage ethanol tolerance assay was determined using the formular below.

$$\text{Percentage ethanol tolerance} = \frac{\text{Ethanol tolerance rate of the isolate}}{\text{Ethanol tolerance rate of the control}} \times 100 \quad (1)$$

2.5. Statistical Analysis

The data obtained from the study were analyzed using IBM Statistical Product and Service Solution (SPSS), version 22. One-way analysis of Variance (ANOVA) with Dunnet test to compare mean across the groups. Mean values with $p<0.05$ were considered statistically significant compared between and within the groups.

3. Results and Discussion

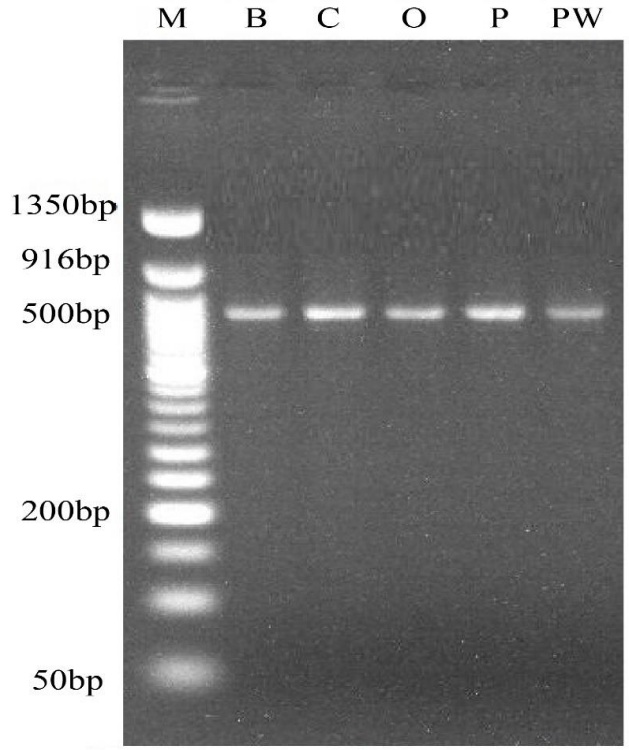
3.1. Identification of the Isolates

Nine recovered yeast isolates included Meyerozyma guilliermondii strain 19, Meyerozyma guilliermondii strain KAS-143, Meyerozyma guilliermondii strain GTi7, Meyerozyma guilliermondii strain ANSe-23, Meyerozyma guilliermondii strain RCPF 1373, Candida tropicalis strain L6, Candida tropicalis strain OBY6, Candida tropicalis strain IDR1000028827 and Kodamaea ohmeri strain TS21(Table 1.0). The recovery of Candida tropicalis from palm wine is in line with the results of Laurence et al. (2022) and Nwaiwu et al. (2016), which isolated the same organism from palm wine and sorghum beer. However, Saccharomyces cerevisiae, Hanseniaspora guilliermondii, Candida intermedia, Kazachstania unispora, Kazachstania exigua, Meyerozyma guilliermondii, Pichia kudriavzevii (Issatchenkia orientalis) and Pichia kluyveri were recovered by Santiago-Urbina et al. (2015) and Anyanwu et al. (2020) from palm wine. Uhitil et al. (2009) and Alabere et al. (2020) also reported the presence several yeast stains, including Candida guillermondii, C. colliculosa, C. albicans, Trichosporon mucoides, Kloeckera spp., Meyerozyma guilliermondii strain 1621, *Pichia* guilliermondii strain PX-PAT, Meyerozyma caribbica strain Kw 1S7Y2, Meyerozyma caribbica strain Y-27400 and Kodamaea ohmeri strain ww1-1 from palm wine, orange, apple, banana (YB) and pineapple (YP) respectively. Meyerozyma guilliermondii is a species of yeast of the genus

Meyerozyma whose asexual or anamorphic form is known as *Candida* guilliermondii. These Candida species, including *C. tropicalis*, have been reported to promote the fermentation process of starch media and can produce ethanol in sufficient amounts (Zulfikar *et al*. 2019; Olowonibi 2017). The presence of these Candida species in palm wine and fruit juices could be of great health concern, as Laurence *et al*. (2022) stated that Candida species are regarded as commensals, but under certain circumstances, such as in immunosuppressed patients, these species can become opportunistic pathogens and can cause candidiasis. Figures 1a & b indicate Gel images showing amplification of the Internal Transcribe Spacer (ITS) of the yeast's isolates (code B, C, O, P, PW FPW1, FPW2, FO1 and FO2). The base pairs of the isolates at 500bp are a confirmation that the isolates were all yeasts, as 500bp has been aligned with yeast DNA base pairs. The phylogenetic tree of the yeast isolates (Figures 2a & 2b) revealed the closely related strain of the yeast isolates.

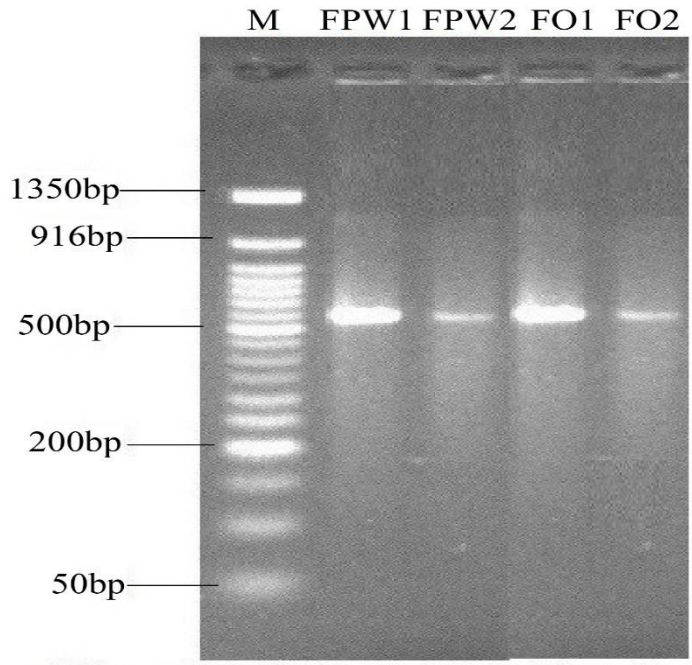
Table 1. Characterization of the yeast isolates

Isolates Code	Source of the Isolate	Predominant Organism's Name	Pairwise Similarities (%)	NCBI Accession Number
B	Banana Juice	Meyerozyma Guilliermondii Strain 19	75.72	OQ137206.1
C	Carrot juice	Meyerozyma Guilliermondii Strain KAS-143	80.95	MG846138.1
P	Pineapple juice	Meyerozyma Guilliermondii Strain ANSe-23	76.79	MG846131.1
PW	Palm Wine	Meyerozyma guilliermondii Strain RCPF 1373	83.53	MT534187.1
FPW1	Palm Wine	Candida tropicalis strain L6	90.61	KF806464.1
FPW2	Palm Wine	Kodamaea Ohmeri Strain TS21	80.88	ON954661.1
FO1	Orange Juice	Candida Tropicalis Strain OBY6	83.7	MH263644.1
FO2	Orange Juice	Candida Tropicalis Strain IDR1000028827	76.74	JN675334.1
O	Orange Juice	Meyerozyma Guilliermondii Strain GTi7	79.65	MT645413.1



Gel image showing amplification the Internal Transcribe Spacer (ITS) of the isolates at 650bp Lane M is a 50bp DNA ladder

Fig. 1(a) Gel image showing amplification of the Internal Transcribe Spacer (ITS) of the yeast isolates (code B, C, O, P, PW)



Gel image showing amplification the Internal Transcribe Spacer (ITS) of the isolates at 650bp Lane M is a 50bp DNA ladder

Fig. 1(b) Gel image showing amplification of the Internal Transcribe Spacer (ITS) of the yeast isolates (code FPW1, FPW2, FO1 and FO2)

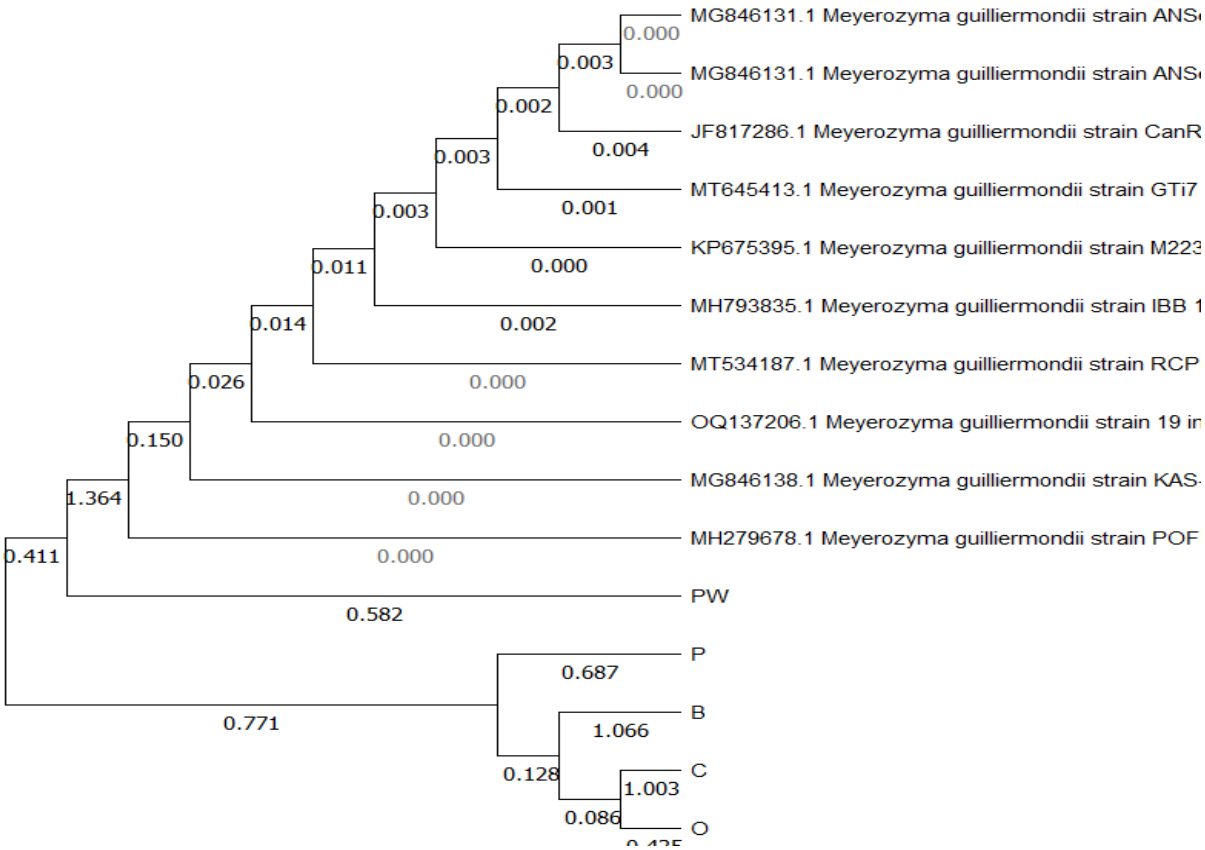


Fig. 2(a) Phylogenetic tree of yeasts isolates (Code B, C, O, P, PW)

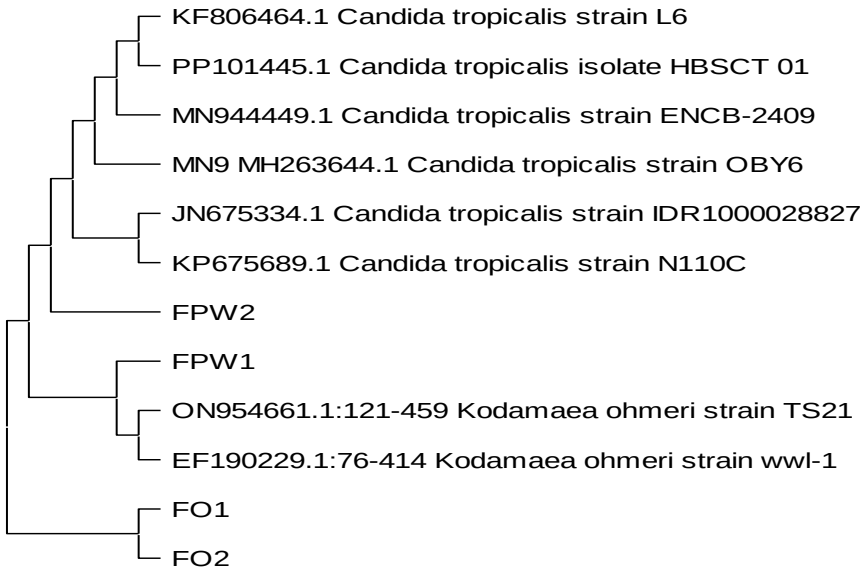


Fig. 2(b) Phylogenetic tree of yeasts isolates (Code FPW1, FPW2, FO1 and FO2)

3.2. Ethanol Tolerance Assay

All the yeast isolates tolerated alcohol up to 16%, but the tolerance rate was higher with Saccharomyces cerevisae (control) in all the concentrations. The rate of tolerance decreased as their concentration was increased (Table 2). *Candida tropicalis* strain IDR1000028827, Meyerozyma guilliermondii strain RCPF 1373 and Candida tropicalis strain L6 tolerated ethanol more than other yeast isolates, but the highest was with Candida *tropicalis* strain IDR1000028827.

Even at 16% concentration, this organism was able to grow, yielding an 80% ethanol tolerance rate. Even though the yeast isolates were able to tolerate ethanol at various concentrations, there was still a significant difference between their rate of tolerance with that of the control (Saccharomyces cerevisae) at $p<0.05$. The ability of these yeast isolates to withstand ethanol gave them an interesting property that makes them exploitable for Industrial application for ethanol production.

The decrease in the ethanol tolerance rate of these yeast strains as the concentrations were increased agreed with the report of Carlsen et al. (1991) that indicated that the accumulation of ethanol in yeast cultures could result in a decrease in the rates of fermentation and the growth rate which can lead to loss of viability. Olowonibi (2017) report that ethanol at higher concentrations is toxic to the yeast, leading to inhibition of the cell's growth due to the destruction of the cell membrane.

The tolerance of ethanol by the isolated yeast strains is in line with the findings of Boudjema et al. (2016), which revealed ethanol tolerance of C. tropicalis Z087B0VS at 12% concentration. Pongcharoen and Miyuki (2018) also reported ethanol tolerance of Candida tropicalis strains at 11-20% concentration. Olowonibi (2017) reported ethanol tolerance of Candida spp at 8 and 10% ethanol concentration but had very low tolerance to 13% concentration.

Table 2. Ethanol tolerance profile of the indigenous yeast

Yeast	**Ethanol Tolerance (%)**					
Isolates	6	8	10	12	14	16
Sacharomyces cerevisae (Control)	100	100	100	100	100	100
Meyer ozyma guilliermondii strain 19	85	83	82	40	28	26
Meyerozyma guilliermondii strain KAS-143	52	33	32	29	28	20
Meyerozya guilliermondii strain GTi7	93	91	86	53	42	30
Meyerozyma guilliermondii strain ANSe-23	86	83	76	72	68	58
Meyerozyma guilliermondii strain RCPF 1373	96	94	83	80	78	72
Candida tropicalis strain L6	96	94	88	85	80	78
Candida tropicalis strain OBY6	88	70	44	36	34	33
Candida tropicalis strain IDR1000028827	97	96	90	88	85	80
Kodamaea ohmeri strain TS21	89	87	60	37	34	24

4. Conclusion

The result obtained from this study revealed the presence of a variety of ethanol tolerance yeast species from the assayed palm wine and fruit juices. However, there was a significant difference between the ethanol tolerance rates of the isolated yeast species with that of in-use commercial Saccharomyces cerevisae. Yet the yeast isolates, especially Candida tropicalis strain IDR1000028827, Candida tropicalis strain L6 and Meyerozyma guilliermondii strain RCPF 1373, which demonstrated above 70% ethanol, can be tried for ethanol production for future use.

Author Contribution Statement

IJO and UOG conceived and designed the research. UFN and IJO conducted experiments. IJO contributed new reagents or analytical tools. UFN and UOG analyzed data. IJO wrote the manuscript. All authors read and approved the manuscript.

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