Isolation and Screening of Yeast Isolates Indigenous Palm Wine for Ethanol Production

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The problem that has been ravaging ethanol producing industries for decades now is the ability of industrial yeast isolates to withstand ethanol production stress conditions while giving out optimal ethanol yeast. Hence, there is need to constantly source for yeast isolates with these qualities. Yeast isolates obtained from aging palm-wine were investigated for their ability to withstand some ethanol production stress conditions. Their growth responses were observed qualitatively at different temperatures, sugar concentrations (up to 200 g/L), and ethanol concentration (up to 20% v/v). A total of 20 yeast isolates were obtained and screened for ethanol stress condition tolerance. *Saccharomyces cerevisiae* SCPW 17 was able to tolerate ethanol production stress conditions with minimal growth at 45°C and 20% v/v ethanol and intensive growth in a medium containing 200 g glucose/L. The identity of *S. cerevisiae* SCPW 17 was determined and confirmed by the analysis of its internal transcribed spacer (ITS1) region of the 18S ribosomal DNA. *Saccharomyces cerevisiae* SCPW 17 exhibited good characteristics needed in yeast isolates meant for ethanol and bio-ethanol production.

Key words: bio-ethanol, growth response, osmotic stress, palm wine, *Saccharomyces cerevisiae*, yeasts

INTRODUCTION

Yeasts are very important in ethanol production through the fermentation of varieties of sugars. They are fungi found as members of the group ascomycetes and basidiomycetes. They have a unique in their budding and fission mode of reproduction (Boekhout & Kurtzman 1996).

Yeasts such as *S. cerevisiae* have been used in alcohol production, especially in the brewery and wine industries, for thousands of years. Obvious reasons being that this yeast gives high ethanol yield (90% theoretical yield), high ethanol productivity, and has a profound ability to withstand high ethanol concentration up to 40 g/L ethanol in the production milieu (Nigam & Singh 2011).

Nowadays, yeasts generally have used to produce bio-ethanol from renewable energy sources. Yeast strains such as *Pichia stipitis, S. cerevisiae*, and *Kluyveromyces fagilis* have been reported as good ethanol producers from various simple sugars types (Mussato et al. 2012; Azhar, et al. 2017). *S. cerevisiae* is ‘generally regarded as safe’ (GRAS) for use in industrial ethanol production for several applications. It tolerates a wide range of pH, which makes it less susceptible to contamination during fermentation.

There are overbearing challenges observed during yeasts sugar fermentation. They include rise in temperature above 35°C, ethanol concentration above 20% (Tofighi et al. 2014), ability to ferment pentose sugars, as well as the ability withstand pretreatment derivatives of lignocellulosic. These are pertinent problems that have been ravaging ethanol producing industries for decades.
now. Yeast growth rate and metabolism increase with increase in temperature until it reaches the optimum value around 30°C. Ethanol concentration increase during fermentation above 10% causes inhibition to their growth and viability (Attfield 1997).

Tofighi and co-authors (2014) suggested that the efficiency of ethanol production on an industrial scale will be increased by using yeasts that are tolerant to inhibitors. The common challenges of yeasts can be overcome by using ethanol-tolerant and thermo-tolerant yeasts. Ethanol fermentation at high temperature is a beneficial process as it selects thermo-tolerant microorganisms and does not require cooling costs (Fonesca et al. 2008). Ethanol-tolerant and thermo-tolerant strains that can resist stresses can be isolated from natural resources such as soil, water, plants, and animals. Hence, this work is aimed at sourcing wild yeast strains with ability to withstand ethanol production stress conditions.

MATERIALS AND METHODS

Source of Isolates
Palm wine was obtained from retailers in Bodija market, Ibadan, Nigeria (7.4358439°N, 3.91923359°E). Isolation was carried out immediately from the palm wine.

Isolation and Culture Method
Yeast extract peptone (YP) medium comprising of peptone (2%, w/v), yeast extract (1%, w/v), and agar (2%, w/v) was used for the isolation of *Saccharomyces cerevisiae* from fermenting palm wine. Triplicates of decimal dilutions (1:10, 1:100, and 1:1000) in sterilized distilled water were inoculated onto YP medium supplemented with glucose (2%, w/v), ethanol (10%, w/v), and chloramphenicol (0.1%, w/v) (modified method of Oliveira et al. 2008).

All plates were incubated inverted at 30°C for 3-5 days in Uniscop incubator (Model 100b012, England). Distinctive yeast colonies were sub-cultured on malt extract agar (MEA) severally to obtain pure colonies. Purified isolates were maintained on MEA slants. The slants were stored at 4°C until use.

Screening Yeast Isolates for High Sugar and Ethanol Tolerant *S. cerevisiae*
Loopful of the yeasts isolated and stored in slants were picked and inoculated into yeast extract broth. These were incubated for 24 h at 30°C. Cell concentrations were adjusted to 10⁶ cell/mL and then used as the inoculum for screening studies.

Fermentation Studies on Yeast Isolates
The fermentation pattern of different sugars including glucose, sucrose, maltose, galactose, mannitol, lactose, and xylose by the isolates was studied. Ten (10) milliliters of 1% (v/v) of these sugars prepared in peptone water were dispensed in test tubes with Durham’s tubes for the detection of gas production. Phenol red was used as the indicator. The test tubes and their contents were autoclaved at 100°C for 10 min. They were then inoculated with 0.1 mL of the yeast isolates previously grown in yeast extract broth above. The inoculated test tubes were incubated at 30°C for 24 h. After incubation, the tubes were observed for acid and gas production (Barnett et al. 1990).

Growth of Yeast Isolates on YP Medium Supplement With 200 g/L Glucose
One milliliter (1 mL) of yeast inoculum was introduced into 50 mL of YP broth medium supplemented with 200 g/L glucose and incubated at 30°C for 72 h. The growth of the isolates in the tubes were observed qualitatively based on the turbidity of the culture medium at the end of the experiment, and this was recorded as low growth, moderate growth, and intensive growth (Guimarães et al. 2006).

Ethanol Tolerance Test
The yeast isolates were examined for ethanol tolerance using the procedure of Kumar and co-authors (2011). One milliliter (1 mL) of each yeast isolate was inoculated into 50 mL YPG broth medium containing different concentrations of ethanol (10%, 15%, and 20%; v/v). The inoculated tubes were incubated at 30°C for 72 h. The growth of the inoculated isolates was examined and recorded as: low growth, moderate growth, and intensive growth depending on the turbidity of the growth medium at the end of the experiment.

Temperature Tolerance Test
The growth response of the yeast isolates at different temperature was studied following the method of Guimarães and co-authors (2006). One milliliter (1 mL) of the pre-cultured yeast isolates containing 10⁶ cell/mL were inoculated into 50 mL YPG broth and incubated at 30, 35, and 45°C for 72 h. The growth of the inoculated isolates was examined qualitatively and recorded as low growth, moderate growth, and intensive growth depending on the turbidity of the growth medium at the end of the experiment.

Identification of the Screened Isolates
Yeast isolates showing remarkable characteristics were selected for identification and further experiments. The cultural, microscopic, and molecular characteristics of the selected isolate were examined in order to identify them.
Morphological Characterization
Purified yeast isolates were transferred to potato dextrose agar plate and incubated for 3-5 days in order to observe its cultural characteristics. The cultural characteristics of the isolates were observed with respect to its appearance, colour, and shape on PDA plates.

Molecular Characterization
DNA Extraction. Cells of the selected isolate grown in a broth medium were centrifuged using Thermo scientific Sorvall Lynx 6000 (USA) super-speed centrifuge at 10000 rpm for 5 min and washed with distilled water (Fietto et al. 2004). They were then re-suspended in a 400 μL solution containing 0.3 mg/mL lyticase and 8 μL/mL β-mercaptoethanol in extraction buffer (1 mol/L sorbitol, 100 mmol/L sodium citrate, 60 mmol/L EDTA, pH 7.0), and incubated in a Clifton waterbath (Model: S/W97719, UK) for 3 h at 37°C. Then, 1 volume of lysis buffer (2% SDS in 50 mmol/L Tris, 10 mmol/L EDTA, pH 8.0) was added and the mixture shaken gently and incubated at room temperature for 10 min, after which 200 μL of 5 mol/L NaCl was added. The suspension was maintained in ice for 2 h. The pellet was harvested by centrifugation at 13,000 rpm for 10 min then suspended in 200 μL of Tris-EDTA buffer, after which the DNA was then de-proteinated with a phenol: chloroform: isoamylalcohol mixture (25:24:1). The aqueous layer was collected and DNA in it was precipitated with ethanol (2 volumes). It was harvested by centrifugation (13000 rpm for 15 min) and then washed in ice-cold 70% ethanol, after which the DNA pellets were dissolved in 60 μL of sterile distilled water.

PCR Amplification and Sequencing of the rDNA Internal Transcribed Spacer Region (ITS). The primers used to amplify the rDNA ITS were ITS1 (CGG GAT CCG TAG GTG AAC CTG CGG) and ITS4 (CGG GAT CCT CCG TT ATT GAT ATG C) as described by White and co-authors (1990). The amplification reaction was done in a 50 μL volume containing 20 pmol of each primer, 300 ng of genomic DNA template, 0.25 mmol/L each dNTP, 1.5 mmol/L MgCl, and 0.5 U of Taq polymerase. The reactions were ran for 34 cycles with denaturation at 94°C for 45 seconds, annealing at 60°C for 1 min, and extension at 72°C for 2 min. An initial denaturation lasted 4 min at 94°C and final extension was at 72°C for 5 min. Amplified products from PCRs were sequenced using automated sequencer (Chromus Biotech, Chennai). The sequence similarity search was done for the rDNA sequences using online search tool called Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). The unknown organism was identified using the maximum aligned sequence through the BLAST search.

RESULTS
Isolation and Identification of Yeast Isolates
A total of 20 yeasts were isolated from fermenting palm wine. The yeast isolates were designated SCPW1 to SCPW20.

Fermentation Pattern of the Yeast Isolates
Table 1 shows the fermentation pattern of the 20 yeast isolates from fermenting palm wine. Their ability to ferment glucose, sucrose, galactose, mannitol, lactose, and xylose was tested. All the isolates fermented glucose producing acid and gas. Sucrose, a disaccharide of fructose and glucose, was also well utilized by all the isolates. Maltose and galactose were not fermented by all the isolates, but those that fermented them did so completely with acid and gas production.

The fermentation pattern of the 20 yeast isolates, as well as their ability to tolerate ethanol production conditions, was studied. This was done in order to screen for a yeast strain

| Carbon sources | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Glucose        | AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG|
| Sucrose        | AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG| AG|
| Maltose        | - | - | AG| AG| - | AG| AG| AG| AG| AG| - | - | - | - | AG| AG| AG| AG| AG| AG| AG|
| Galactose      | - | - | AG| AG| - | AG| AG| AG| AG| AG| - | AG| - | AG| AG| AG| - | - | - | - | - |
| Mannitol       | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Lactose        | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Xylose         | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Key: A= acid production; G= gas production; - = no acid and gas production.
that can withstand various ethanol production conditions
(Table 2, 3, 4, and 5). Isolate 17 grew moderately in a
medium supplemented with 200 g/L glucose and was able
to withstand 20% v/v ethanol and temperature of 45°C.
The isolate was able to survive in a medium containing
10% w/v glucose that was incubated at 45°C up to 36 h,
Identification of Isolate
The isolate exhibiting remarkable characteristics was identified after cultural, morphological, and molecular studies. It was confirmed to be *Saccharomyces cerevisiae* SCPW17 after partial 18S rDNA sequencing. The phylogenetic relationship of this isolate is shown in Figure 2. *Saccharomyces cerevisiae* SCPW17 is closely related to *S. cerevisiae* CBS 1171 with 100% sequence identity.

CONCLUSION
The 20 yeast isolates obtained from fermenting palm wine were predominantly *Saccharomyces* spp. Others included members of the genera *Pichia* and *Candida*. Nwachukwu and co-authors (2006) reported the isolation of mainly *Saccharomyces cerevisiae* from aged palm wine. The prevalence of *Saccharomyces* spp. in fermenting palm wine can be attributed to the fact that they can withstand the increasing ethanol content of aging palm wine which could be as high as 10% (v/v) (Oliveira et al. 2008). Nevertheless, the nutritional composition of fresh palm wine literally makes it a good medium for growth of many other microorganisms. These organisms could originate from the tapping equipment, the environment, the plant surfaces, and even the containers (Santiago-Urbina & Ruiz-Teran 2014), but most are subsequently eliminated as the palm wine ages. In fact, palm wine is considered safe for drinking as the ethanol present is sufficient to eliminate pathogenic and opportunistic microorganisms that maybe present in palm wine (Bisson & Butzke 2007).
Saccharomyces cerevisiae SCPW17 – out of the 20 yeast isolates – was able to grow at 40°C moderately at 200 g/L glucose and 200 mL/L ethanol, which makes it a potential strain for ethanol production from mannan biomasses. This is similar to the Saccharomyces cerevisiae (KY2) reported by Khaing and co-authors (2008) that was able to survive in medium supplemented with 20% (v/v) ethanol. Kumar and co-authors (2011) also reported alcohol resistant yeast S. cerevisiae from Toddy samples harvested from a local area of Sivakasi in Virdhunaga District, India. Similarly, Guimarães and co-authors (2006) reported the isolation of Saccharomyces cerevisiae strains that could withstand 150 g/L ethanol concentration and osmotic stress with sucrose at 200 g/L in their growth medium, and could grow at temperature of 45°C for 72 h. According to Casey and Ingledew (1986), some S. cerevisiae strains can withstand high ethanol concentrations above 20% (v/v). They also have good tolerance to lignocellulose derived inhibitors and high osmotic pressure. To support this assertion, Zuzuarregui and Olmo (2004) suggested that the resistance to stress conditions by S. cerevisiae could be linked to the presence of genes that code for such resistance. Saccharomyces cerevisiae strains from palm wine are said to be more ethanol tolerant than those from red wine or sweet stem sorghum juice (Bulawayo et al. 1996).

REFERENCES


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