ISOLATION AND CHARACTERIZATION OF ALCOHOL-TOLERANT *SACCHAROMYCES CEREVISIAE* FROM PALM WINE (*RAFFIAPALM*)

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Abstract
In this present study, fresh palm wine was collected and stored at room temperature 28±2°C prior to yeast's isolation. The yeast isolates were identified based on morphological and fermentation tests. Screening of yeast isolates for ethanol tolerance was monitored in yeast extract peptone glucose medium (YEPG). Estimation of ethanol concentration was carried out by distillation method. Optimization of cultural conditions such as initial sugar concentration, pH, incubation period and initial peptone concentration on Saccharomyces cerevisiae for bioethanol production in YEPG were monitored. The highest yeast counts 30 CFU/mL was obtained in sample collected from Abusoro. The isolated and identified yeasts were Sacch. cerevisiae. The Sacch. cerevisiae tolerated up to 15% ethanol with varied viability ranged from 1.5 to 8.0 in all the samples. Incubation at 72 hours yielded an optimum ethanol production in the medium. The maximum ethanol production at 6% initial sugar concentration and 0.4% peptone was obtained when compared with the control. Optimum pH 6.0 was attained for ethanol production by the yeast isolate. The maximum amount of ethanol produced after distillation was 18.21%. Utilization of ethanoltolerant Sacch. cerevisiae for industrial fermentation of sugar-enriched substrates for ethanol production would lessen distillation costs and at the same time improves its yield.

Keywords: Palm wine, *Saccharomyces cerevisiae*, alcohol, cultural conditions

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INTRODUCTION

Palm wine is a popular traditional alcoholic beverage produced by natural fermentation of sap of various palms (*Elias guineensis* or *Raphia* spp) usually consumed by more than ten million people in the West (Santiago-Urbina *et al*., 2014). The drink is a rich nutrient medium containing sugars, protein, amino acids, alcohol and minerals (Ezeagu and Fafunso, 2003). The unfermented raphia palm sap is clean, sweet; colorless syrup containing 10-16.5% (w/v) sugar (mainly in the form of sucrose) fermented to ethanol and other minor constituents by a complex mixture of wild yeasts and bacteria. The naturally fermented raphia palm wine contains about 5 to 6% (v/v) ethanol (Nwokeke, 2005).

The negative influences of fossil oil products like gasoline on ecosystems and microbial balance of importance microorganisms coupled with the growing concerns on climate change, partly brought about by excessive carbon dioxide emission of motor vehicles fueled by fossil oils have paved way for the utilization of cheaper renewable fuels like ethanol worldwide (Irene *et al*., 2009). Ethanol is an alternative fuel used in automobiles and as a better substitute to octane enhancers (benzene, butadiene and lead) known to be obnoxious to the environment. It has really attracted global attention with lots of efforts being put in place for its large scale production through microbial fermentation to meet industrial demands.

The microbial fermentation of sugars or organic substrates enriched with glucose with a view to produce bio-ethanol results in a variety of products to include alcohol, carbon dioxide, water, syrup, molasses, stillage and other alcohol (Kumar *et al*., 2011). In order to reduce the cost of bio-ethanol production, there is a continuous search for efficient strains of *Saccharomyces cerevisiae* and other closely related yeast strains. The desired qualities in yeast strains for high ethanol production are efficient aerobic and anaerobic metabolic capabilities, ethanol tolerance, thermotolerance

and resistance to killer yeasts. Uses of efficient yeast strains with higher ethanol tolerance potential will in no doubt reduce distillation costs and hence the profitability of the overall process (Chandrasena *et al*., 2006). The choice of yeast for ethanol production is baker's yeast *Sacch. cerevisiae*. During the pretreatment of hemicelluloses to simple sugarsby chemical or biological methods, some inhibitory compounds are formed, and *Sacch. cerevisiae* has been confirmed to be one of the most inhibitor tolerant microorganisms (Kumar *et al*., 2011). Therefore, the present findings aim at the characterization and screening of yeast strains from palm wine with a view to obtain higher alcohol-tolerant *Sacch. cerevisiae* for bio-ethanol production.

MATERIALS AND METHODS

Sample Collection and Yeast Isolation

Fresh palm wine were collected in sterile containers from four different locations in Akure South Local Government Area of Ondo State, Nigeria and transferred to laboratory immediately. The isolation of yeasts from the samples was done by serially diluted 1mL of the sample into 9mL of sterilized distilled water inside the test tubes. From the diluents, 0.1ml was aseptically pipette and dispensed into sterilized Petri dish, pour plated with sterilized molten yeast extract agar (YEA). After incubation at 28±2^{o}C for 48 hours, the colonies were counted. Pure culture of the yeast isolates were obtained by repeated streaking on YEA and stored in a refrigerator at 4^{o}C for further analysis (Atlass, 2010). Yeast isolates was identified by means of morphological examination and sugar fermentation tests.

Sugar Fermentation Test

The sugar characteristic fermentative ability of the yeast isolates served as guidelines for identification purpose. The fermentative ability was profiled using different sugars involving hexoses (glucose, fructose, galactose), and disaccharides (maltose, lactose, and sucrose). The sugars were separately prepared in a test tube containing 5ml Phenol red broth medium, inoculated with the isolates accordingly and incubated at 30°C for 48 h. afterwards, the tubes were observed for colour change. Colour change from red to yellow is an indication of sugar fermentation and signal reduced pH due to acid production (Barnett *et al*., 1990).

Ethanol Tolerance Test

Standardized yeast cells were inoculated in yeast extract peptone glucose (YPG) broth with varied ethanol concentration ranged between 2.5 and 15.0%, and incubated at 30°C for 2 days. After incubation, the population of yeast cells wasenumerated by plating serially diluted yeast broth supplemented with varied alcohol concentrations on YPG agar medium and then incubated. The colonies emerged from the agar medium were observed and recorded as colony forming unit (CFU/mL).

Ethanol Assay by Potassium Dichromate and Sulphuric Acid Method

One milliliter (1mL) of the cell free culture obtained after centrifugation was made up to 5ml with distilled water, followed by the addition of 1mL of $K_2Cr_2O_7$ solution and 4mL concentrated H_2SO_4 solution was further added. The absorbance of color developed from the preparation was read at 660nm in VIS spectrophotometer (AXION 721). Blank is prepared by replacing equal amount of culture supernatant with distilled water. Ethanol production was assayed by comparing the absorbance of obtained from the sample with alcohol standard graph (Kumar *et al*., 2011).

Distillation of Ethanol

The fermentation process was carried out in YPG medium under optimized process parameters in 1000ml Erlenmeyer flask. The optimized cultural conditions are pH- 3, temperature-30^{o}C and 72 hr of incubation. After incubation, ethanol was distilled from the fermented broth by fractional distillation (Kumar *et al*., 2011).

Process Parameters Optimization for Ethanol Production

The effect of initial sugar concentration on ethanol production was tested by supplementing the alcohol production medium with varied glucose concentrations. After incubation at 30°C for 48 hr, the samples were collected and assayed ethanol concentration

(Roukas, 1996). The fermentation flasks inoculated with yeast strain was incubated for 120 hrs, and at an interval of 24 hrs sampling was carried out to evaluate the amount of ethanol generated (Tahir *et al*., 2010).

The effect of different pH values on ethanol yield was tested adjusting the pH of the YPG medium to cover a pH range from 2.5 to 5.5 (all adjustments were made before sterilization by either NaOH or HCl). After inoculation with yeast strainand incubation, the ethanol concentration in the production medium was estimated (Tahir *et al*., 2010). The effect of initial peptone concentration on ethanol yield was also tested by varying the concentration of peptone (0.0, 0.1, 0.2, 0.3, 0.4, and 0.5%) in YPG. The overnight culture of yeast cells were inoculated and incubated at 30°C for 48 hrs. After incubation samples were drawn and tested for concentration of ethanol (Wang *et al*., 2007).

RESULTS AND DISCUSSION

Total Yeast Counts

Table 1 shows the total yeast counts from palm wine collected from different locations. Sample collected from Igbara-Oke had the highest yeast count of 30.0 CFU/mL, while the lowest value of 14.0 CFU/mL was obtained from the sample collected from Abusoro. Palm wine is enriched with nutrients and it is considered as an excellent substrate for the growth and proliferation of microorganisms such as bacteria yeasts (Karamoko *et al*., 2012; Santiago-Urbina *et al*., 2013). Boboye *et al*. (2008) and Gidado *et al*. (2014) had earlier reported varied yeast populations and the isolation of yeast *Sacch. cerebisiae* from palm wine. The variation observed in the yeast isolated from the fermenting palm wine can be attributed to the source and time of collection, methods of tapping, storage container, environmental conditions, processing, freeze-thaw, osmotolerance and human activities. The occurrence of yeast *Sacch. cerevisiae* as predominant fermenting organism in many fermented products has been reported (Kumar *et al*., 2011; Preeti *et al*., 2014, Umaru *et al*., 2014). The presence of yeast in the palm wine further buttress the facts that palm wine is richer in fermentable sugars that allowed yeast to thrive which often led to acid production after fermentative activity (Essien *et al*., 2011).

Morphological and Microscopic Identification of Yeast Isolates

The morphological and microscopic identification of yeast isolates is shown in Table 2. The yeast,*Sacch. cerevisiae* was common to all the samples obtained from different locations. The morphological and microscopic observation of the yeast isolates under the microscope showed spherically or ellipsoid-shaped cell/bud cell of ascospore. The morphologic features of the yeast isolate was confirmed by mounting on glass slide and stained with crystal violet. On microscopic examination, ascospores were seen (result not shown). Physiologic sugar fermentative profiling of isolate was carried out with carbon substrates such as galactose, glucose, sucrose, maltose and raffinose (Table 2). The isolate was tentatively identified as *Sacch. cerevisiae* grounded on its characteristic cellular morpho-physiologic and sugar fermentative attributes. The colour change from red to yellow after 48 h of fermentation might be attributed to acid production, and this agreed with the report of Kumar *et al.* (14).

Table 1. Total yeast counts

Sample source	Yeast count (Cfu/mL x 10^2)
OK	20
IG	30
AB	14
OD	23

Key: OK = Oke-Odo, IG = Igbara-Oke, AB = Abushoro, OD = Odosha

Table 2. Morphological and microscopic identification of yeast isolates

Test	Sample source			
	OK	**IG**	**AB**	**OD**
Cell size/arrangement	F/C/S	F/L/C	F/C/S	F/L/C
Cell shape	Ovoid	Ovoid	Ovoid	Ovoid
Colour	Blue	Blue	Blue	Blue
Sugar fermentation				
Sucrose	+	+	+	+
Glucose	+	+	+	+
Galactose	+	+	+	+
Maltose	+	+	+	+
Xylose	-	-	-	-
Raffinose	+	+	+	+
Arabinose	-	-	-	-
Lactose	-	-	-	-
Probable isolates	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*

Key: + = Fermentable, - = Non fermentable, F= fat, C = chains, S = short

Ethanol tolerance of Sacch. cerevisiae

The effect of different ethanol concentrations on the population of *Sacch. cerevisiae* is represented (Table 3). The population of all the yeast strains evaluated decreased with increase in ethanol dose supplemented in the YPG broth when compared with the control experiment where there was no ethanol incorporation. At 15% ethanol supplementation, yeast strain from Igbara-Oke designated as IG has the highest yeast load while the least count was recorded for the strain coded as OD. Ethanol tolerance is one of the criteria for selection of strains and unique properties of the yeast that makes it exploitable for industrial ethanol production. The ethanol tolerance of the yeast isolated from palm wine could depend on their ability to tolerate the physicochemical conditions and inherent genetic make-up. The use of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermentation product (palm wine) would reduce distillation costs and hence the profitability of the overall process (Chandrasena *et al.*, 2006). The ethanol tolerant yeast from palm wine between 15 and 20% has been favorably used in brewing (Nwachikwu *et al*., 2006).

Table 3. Ethanol tolerance and viability of *Sacch. cerevisiae* for bioethanol production

	***Sacch. cerevisiae* (CFU/mL x 10^5)**			
Amount of ethanol (%)	**OK**	**IG**	**AB**	**OD**
0.0	6	7	6.5	8
2.5	2.4	3.5	1.7	2.6
5.0	3	2.7	2.4	1.8
7.5	1.9	1.9	4	2.1
10.0	1.5	2	1.6	2.1
12.5	2.2	2.4	1.8	1.8
15.0	2.4	4	2.7	2.3

Key: OK = Oke-Odo, IG = Igbara-Oke, AB = Abushoro, OD = Odosha

Manikandan *et al.* (2010) reported that most of the ethanol producing yeast strains isolated from palm wine could tolerate ethanol concentration from 10 to 12%. Kumar *et al.* (2011) and Preeti *et al.* (2014) reported 13% and 7% ethanol concentrations respectively for *Sacch. cerevisiae* growing in toddy. Similarly, ethanol tolerance of selected strains of *Sacch. cerevisiae* had also been confirmed by Nwanchukwu *et al.* (2008) who reported 15% and 10% ethanol concentrations in a separate studies.

Optimization process parameters for bio-ethanol production

Figure 1 shows the effect of initial sugar concentration on ethanol production by *Sacch. cerevisiae*. Production of ethanol by *Sacch. cerevisiae* varied with the doses of sugar added to the production media. Maximum yield of ethanol was reached at 6% sugar supplementation while the least was obtained with 1% sugar. Sugar is a vital carbon substrate for microbial fermentation; microorganisms participating in sugar utilization derive energy for subsequent metabolism. The yield of ethanol is greatly influenced by fermentation process and the physiological state of the organisms initiating such a process. The growth of yeast is enhanced by the presence of high sucrose concentration in palm wine and their ability to ferment sugars. According to the findings of Govindaswamy and Vane (2010), 5% (w/v)glucose was observed to be the best concentration out of all the concentrations tested in that its supplementation resulted into maximum ethanol yield. In another experiment conducted by Mishima *et al.* (2008), initial sugar concentrations 30g/l and 33g/l yielded maximum ethanol 14.4 g/l and 14.9g/l respectively. Different best initial sugar concentrations have been reported by many researchers; Kumar *et al.* (2011) reported 4% with the highest yield 15g/l while Manikandan *et al.* (2010) reported 20% with maximum ethanol yield (40 g/l) from *Sacch. cerevisiae* isolated from toddy.

Ethanol yield increased with increase in incubation time and reached optimum at 72 hours (Figure 2). Incubation beyond 72 h resulted into a decline in ethanol yield. The decrease in the yield of ethanol by the producing organism beyond optimum incubation time might be due to either depletion of sugar and essential growth promoters from the production medium or accumulation of toxic metabolites generated during metabolic activity (Akinyele *et al.*, 2013). Mishra *et al.* (2011) achieved maximum ethanol production when *Sacch. cerevisiae* was cultured on orange peel for 72h. Similar result was obtained and reported by Ferrai *et al.* (1992) when *Pichia stipitis* was cultivated on eucalyptus wood hemicelluloses for 72h with a view to produce ethanol maximally. Maximum 16-19 g/l ethanol was produced by *Kluyveromyces marxianus* CECT 10875 on lignocellulosic biomass as substrate in simultaneous saccharification and fermentation process at 72h of incubation (Ballesteros *et al.*, 2004).

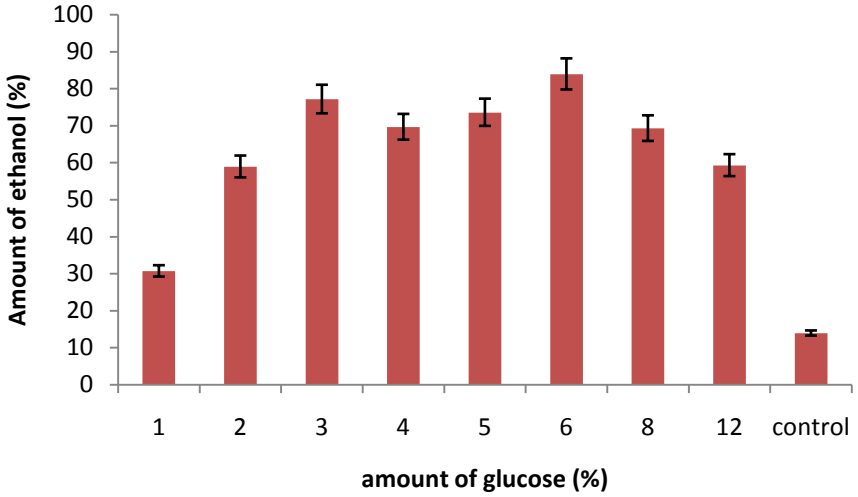

Figure 1. Effect of initial sugar concentration on bioethanol production

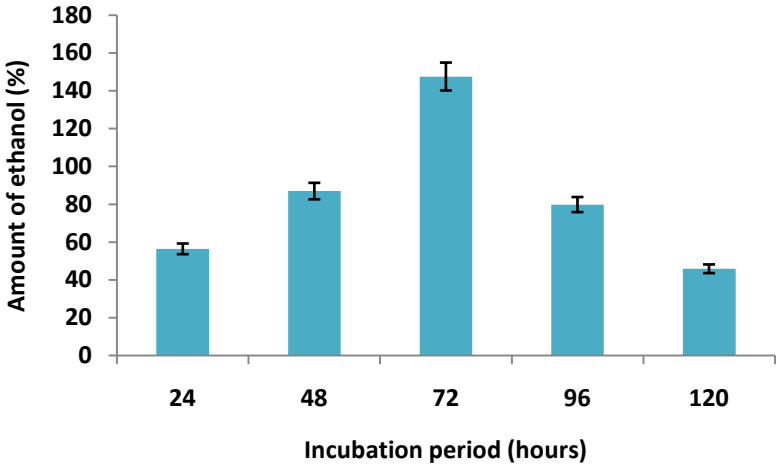

Figure 2: Effect of incubation period on bioethanol production

The findings of Wang *et al.* (2007) also corroborated the result obtained from this study; 72h was the best fermentation time for ethanol production when hydrolysed sugar from orange peel was fermented by *Sacch. cerevisiae*. Kumar *et al.* (2011) reported 72 h as the optimum incubation time for bioethanol production from alcohol resistant yeast *Sacch. cerevisiae* from toddy. Figure 3 shows the effect of pH on bioethanol production. In this present study, highest ethanol (230.7%) production was obtained at pH 6.0 and at pH 8.0 bioethanol decreased by 69.93%. The growth and metabolic activity of each microorganism is permitted within specific a pH range; pH is pivotal to a successful fermentation process. In this study, increase or decrease in pH on either side of the optimum led to low ethanol yield. Metabolic pathways and growth of yeast is retarded in more acidic and basic conditions (Willaert and Viktor, 2006; Fakrudin *et al.*, 2013). It is an established fact that yeast possessed ability to thrive and survive in slightly acidic medium but in high acidic condition their metabolism becomes impaired, therefore, an optimum pH must be selectedfor any bio-product formation. Selection of pH beside the pH range of the intended organism can affects the ionization of essential active site of required enzyme molecules that are involved in substrate binding during catabolism (Akinyele *et al.*, 2013). In separate studies, Mohanty *et al.* (2009), Togarepi *et al.* (2012) and Ashok *et al.* (2014) reported a pH 6.0 as the optimum pH among the tested pH range for bioethanol production from mahula (*Madhuca latifolia* L.) flowers, Ziziphus mauritiana fruit pulp and supplemented sweet potato respectively.

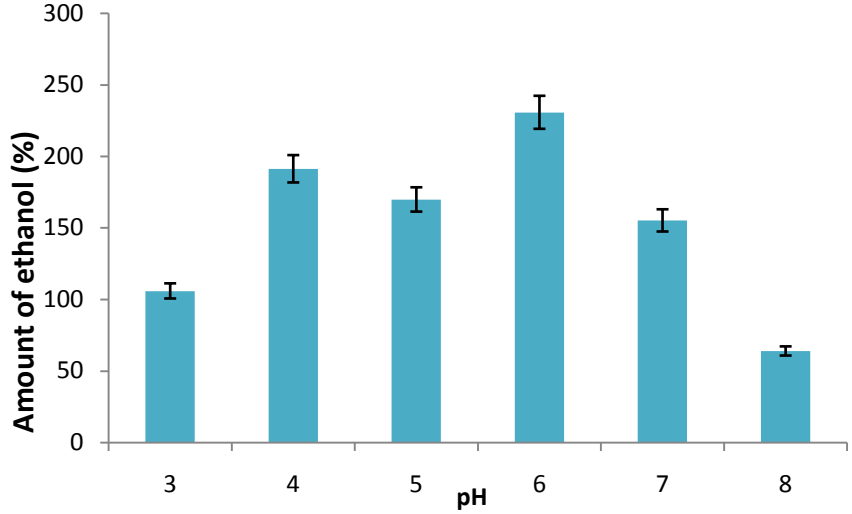

Figure 3: Effect of pH on bioethanol production

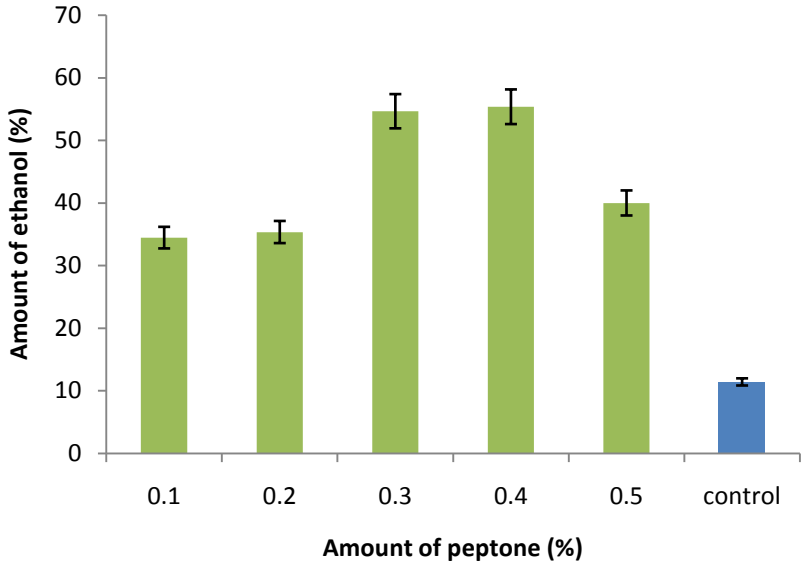

Figure 4: Effect of peptone on bioethanol production

The reports of Kundiyana *et al*. (2010) was quite lower from the value obtained in this study, optimum pH 4.3 was reported for bioethanol production by *Sacch. cerevisiae* cultivated on sorghum juice. Different optimal values, pH 6.5, pH 4.5 and pH 5.5 were reported for maximum ethanol production by Udhayaraja and Narayanan (2012) by using *Sacch. cerevisiae* as a producer.

The effect of initial peptone concentration on ethanol production was evaluated to know the best concentration for biethanol production (Figure 4). The production of ethanol increased with increase in the concentration of peptone and reached maximum production at 0. 4% peptone incorporation, beyond this value, a decline in ethanol yield was observed. The importance of peptone to microorganisms is not negotiable to their growth; it is a mixture of proteins and amino acids derived from plant and animal tissues. According to Fundora *et al.* (2000), incorporation of 1% peptone speed up the rate of fermentation and hence enhanced ethanol yield. In a research conducted by Ashok *et al*. (2014), a lower peptone concentration 0.2% supplemented with sweet potato flour was reported to support maximum ethanol production at different incubation time. The YEPG medium was optimized based on the best cultural parameters obtained from this study, and the ethanol yield was 18.21% after distillation.

In conclusion, *Sacch. cerevisiae* from palm wine demonstrated a good level of ethanol tolerance which is a desirable attribute for an organism to be exploited for bioethanol production. In addition, an appreciable ethanol yield of 18.21% was obtained after distillation. The optimal process parameters for bioethanol production by *Sacch. cerevisiae* isolated palm wine were 72hrs incubation time, pH 6.0, peptone 0.4% and 6% glucose supplementation.

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