Effect of *Sacoglottis gabonensis and Alstonia boonei* on the Fermentation of Fresh Palm Sap by *Saccharomyces,Cerevisiae*

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Abstract: *Saccharomyces cerevisiae* isolated from fresh Raphia palm wine was cultured in *Sacoglottis gabonensis* (0.625%) treated medium, *Alstonia boonei* (0.50%) treated medium and untreated medium. Concentrations of plant products used were predetermined as the optimal concentrations of the plant product that had a preservative effect on fermenting palm sap. Flocculation ability, ethanol production, ethanol tolerance, osmotolerance and invertase activity of the yeast isolates in each of the cultures were determined and compared. Alcohol content (6.42%v/v) of untreated sample was significantly lower (p≤ 0.05) than alcohol content (12.07%v/v) of sample treated with *S. gabonensis* and *A. boonei* (11.53% v/v). The two plant products enhanced ethanol tolerance and osmotolerance of the yeast. The flocculation as well as invertase activities of the yeast were however reduced by the two plant products.

Key words: *Sacoglottis gabonensis, Alstonia boonei, Saccharomyces cerevisiae,* fermentation, palm wine, flocculation, osmotolerance, Invertase, ethanol

INTRODUCTION

Palm wine is a traditional fermented alcoholic average obtained from the sugary sap of palms (family: Palmae). It is defined as the phloem exudate from the palm tree when the emerging palm inflorescence is excised[1] . It is a refreshing beverage widely consumed in the tropics particularly in West Africa, Asia and South America[2]. In some parts of Nigeria, its production has developed into small-scale industries.

Fresh palm sap is a sweet, clear, colorless juice, which has high sugar content. Fermentation of the sugars and other nutrients by the endogenous micro flora of the palm results in the rapid deterioration of the arganoleptic quality of the juice as most of the sugars are converted into ethanol and organic acids. The wine becomes unacceptable to most consumers after about 24 h of production. To prolong the shelf life of fresh palm wine, some local traditional wine tapers add the pulverized dust of the tree bark of *S. gabonensis* to the fermenting palm sap. The optimal concentration of this plant product added has been determined to be 0.625%[3]. Faparusi and Basir[4] had earlier reported that the tree bark of *Alstonia boonei* is also added to fresh palm juice to preserve it.

Palm wine is rich in non-pathogenic microorganism hence the wine is usually consumed without clarification and stabilization yet causes no harm to the consumers[5].

Uzochukwu *et al* .[6] reported that palm wine results from a yeast/lactic acid fermentation of the sugary sap of the palms. The palm wine contains several species of yeasts most, of which have a high potential for industrial application. Okoli and Ezenkwe[7] reported that *Saccharomyces* species constitute about 50% of the total yeast population, which Okafor[4] had earlier reported that 70% of the *Saccharomyces* yeast is of the species *S. cerevisiae*. Some of these yeasts have been found useful as food for man and feed for animals. Others have high potential for industrial applications[8,9]. High sugar and ethanol tolerant yeasts have been isolated from palm wine[10].

Despite the considerable amount of work done on the preservative effect of *S. gabonensis* on palm wine, little or no information exist on its effect on the micro flora. It is important to determine its effect on the yeasts *S. cerevisiae*, which is most often utilized for industrial fermentations and constitute about 70% of the total yeast population in palm wine. Such information would reveal the potential of this plant product for application in industrial fermentations and can be extended to other foods and beverages. The present study reports on the effect of *S. gabonensis* and *A. boonei* on some characteristics of *S. cerevisiae* relevant to industrial fermentation. The result would provide useful information on other possible benefits that are derivable from the use of these plant products.

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MATERIALS AND METHODS

Yeasts strains and culture media: Yeasts isolates were obtained after ten fold serial dilution, from fresh palm wine samples collected at random from different locations of Akwa Ibom and Abia State of Nigeria using conventional mycological techniques[11]. Reference yeast was a bottom fermenting brewer's yeast, *S. cerevisiae* (var. uvarum) and was obtained from Champion Breweries Plc, Uyo, Akwa Ibom State, Nigeria. Growth and maintenance medium was Yeast Peptone Dextrose (YPD) agar containing the following per litre, yeast extract (Sigma) 10 g, peptone (Oxoid) 20 g, glucose (BDH) 20 g and agar (Oxoid) 20 g. Discrete colones that grew on the plates were aseptically picked on the basis of morphology and purified by sub culturing and then incubated at 4°C the ability of the isolates to utilize certain sugars for growth was used as an identification criteria[12]. Isolates identified as *S. cerevisiae* were used for the studies.

Preparation of materials: The tree barks of *S. gabonensis* and *A. boonei* were obtained from an herbal dealer in Umudike near Umuahia, Abia State, Nigeria and taxonomically citified. They were sun dried, cleared manually and pulverized to fine powder using an electric blender to pass 0.5 mm aperture. The optimal concentrations of *S. gabonensis* (0.625%) and *A. boonei* (0.50%), introduced into the sample in the powdery form, were used in the present study.

Flocculation Ability: The effect of *S. gabonensis* and *A. boonei* on *S. cervisiae* isolated from palm wine was studied using the method described by Ezeronye and Okerentugba[13]. The medium used was YPD broth containing in gL^{-1} yeast extract 10, peptone 20 and glucose 20. Sterilized medium (200 mL) were dispensed into four 250mL Erlenmeyer flasks. One of the flasks was treated with *A. boonei* (0.5% v/v) while another are was treated with *S. gabonensis* (0.625% v/v). Both flasks were shaken vigorously and allowed to stand for about 30 min to ensure proper extraction of the plant product. The last two flaks contained no plant product. However one of them was inoculated with 10^6 cells/mL of the bottom fermented brewer's yeast (*S. cerevisiae* var. uvarum), which served as a standard. The other flask as well as the ones containing plant products was inoculated with *S. cerevisiae* isolated from palm wine. The flasks were closed with traps to allow CO_2 to leave. The Optical Density (OD) readings of the cultures were taken immediately at 540 nm using a spectrophotometer, (LKB-Biochrom Novaspec, Model 4049, England) against the medium blank having the same composition as each culture. The flasks were

incubated at 30°C in shaker incubator (Gallenkamp) at 200gmin. sample were taken aseptically from each culture after every 2 h, for 12 h and the turbidity of the cultures were monitored using a spectrophotometer.

Ethanol production and tolerance: The method described by Miklos and Sipiczki[14] and modified by Ezeronye[15] was adapted to investigate the effect of the plant product on alcohol production and ethanol tolerance of *S. cerevisiae* isolated from palm wine. Inoculums for fermentation and ethanol tolerance test was grown in YPS broth (yeast extract 0.5%, peptone 1%, sucrose 30% pH 4.6) in culture tubes containing 5 mL of the medium and incubated at room temperature. The culture was grown to stationary phase (15-18 h old) before 10^6 cells/mL was inoculated into three 300 mL Erlenmeyer flasks each containing 200 mL of the some medium. A boonei (0.5%) was added to one of the flasks while 0.625% *S. gabonensis* was added to the second flask. The third flask contained no plant product and served as control. Plant products were added after the sterilization of the medium, before the yeast was inoculated. The flasks were closed with gas traps to allow 10_2 to leave and then incubated at 30°c in a shaker incubated (Gallenkamp) at 200 g min^{-1}. samples were taken after 72 h and the alcohol content determined using the specific gravity method.

Ethanol tolerance was investigated by supplementing sterilized medium with different concentrations (0,3,6,9,12 and 15%) of ethanol. The experiment was set up in 3 units of six Erlenmeyer flask (300 mL), with each unit containing the six levels of ethanol. One unit of the experiment contained in addition, *A. boonei* (0.50%), while another unit contained *S. gabonensis* (0.625%), in each of the flasks. The test unit contained no plant product and served as control. The content of each flask was shaken vigorously and allowed to stand for about 30 min to ensure proper extraction of the plant products.

An aliquot (10^6 cell/mL) of the stationary phase culture (*S. cerevisiae* isolated from palm wine) was inoculated into all the Erlenmeyer flasks. The initial Optical Density (OD) of each culture was taken at 540 nm using LKB-Biochrom Novaspec spectrophotometer (Model 4049, England). Un-inoculated medium corresponding to that of each culture was used as the blank. The flasks were then plugged with cotton wool and incubated at 30°C in a shaker incubator (Gallenkamp at 200g min^{-1}. samples were taken aseptically from each culture after every 2 h for 12 h and the absorbance read in the same way. The experiment was carried out in duplicates. Mean values were used to determine the maximum specific growth rate using the Monod kinetic growth model[16].

Osmotolerance: Effect of *A. boonei* and *S. gabonensis* on the ability of the yeast isolate to tolerate sugar was studied using YPD broth containing (gL^{-1}) yeast extract 10, peptone 20 and glucose added in concentrations of 100,150,300,400 and 500 into five different Erlenmeyer flasks[17]. Three experimental units were set up. Each experimental unit consisted 5 Erlenmeyer flasks (300 mL) capacity), contained in addition, *A. boonei* (0.50%), while another unit contained *S. gabonensis* (0.625%), in each of the flasks. The third unit contained no plant product and served as the control. The flasks containing plant product were shaken vigorously and allowed to stand for about 30min to ensure extraction of the plant products. The palm wine yeast isolate (10^6 cell/mL), was inoculated into all the Erlenmeyer flasks, each containing 200 mL of YPD. The initial OD readings of all the cultures at 540nm were determined using a spectrophotometer (LKB-Biochrom Novaspec, model 4049, England). Uninoculated medium having the same composition with each culture was used as blank. The flasks were covered with gas traps to allow CO_2 to leave and then incubated at 30°C in a shaker incubator (Gallen Kamp) at the revolution of 200gmin. Samples were taken aseptically from each culture after every 2 h, for 12 h and the OD readings was the recorded. The experiment was carried out in duplicate. The maximum specific growth rates were determined from the mean values obtained, using the Monod kinetic growth model.

Invertase activity: The effect of *S. gabonensis* and *A. boonei* on the invertase activity of *S. cervisiae* isolated from palm wine was studied using the method reported by Ekunsanmi and Odunfa[18] with slight modifications. The yeast isolate was inoculated by single streaking on YPD agar slants, in three sterile bottles, each containing (gL^{-1}) yeast extract agar 10; peptone 20; glucose 20. The cells were grown for 48 h at 30°C. One bottle contained *S. gabonensis* (0.625%) in the medium. The third bottle contained no plant product and served as control. Pouring sterile distilled water into the slants and gently scrapping with sterile loop was used in harvesting the cells from each culture. They were then washed and centrifuged. The harvested cells (0.1 g wet weight) from each culture were re-suspended in 10 mL of acetate buffer. One milliliter of the cell suspension was added to 2 mL of 4% sucrose solution, prepared using the same buffer and incubated for 5min at 30°C. The amount of reducing sugar released was determined using dinitrosalicylic acid (Bernfield, 1951). One unit of invertase activity was defined as the amount of enzyme in 1mL of the solution in 5min at 30 °C, pH 5.0. The experiment was carried out in duplicate. Mean values were reported.

RESULTS AND DISCUSSION

The morphology and fermentation characteristics of yeasts isolated from palm wine(Table 1 and Fig.1. Effect of plant). The yeast isolates include *Saccharomyces* sp, *Candida* sp. and *Schizosaccharomyces* sp. 60% of yeasts (estimated by percentage occurrence of colonies) isolated from the palm wine were identified as *Saccharomyces*. This agrees with earlier reports on the microbiology of palm wine, which indicated that the predominant yeast microflora, were *Saccharomyces* species[7,8]. Amanchukwu *et al.*,[19] also isolated a hydrocarbon degrading strain of *Schizosaccharomyces pombe* from the Nigeria palm wine and subsequently used the isolate for production of single cell proteins on hydrocarbon substrate.
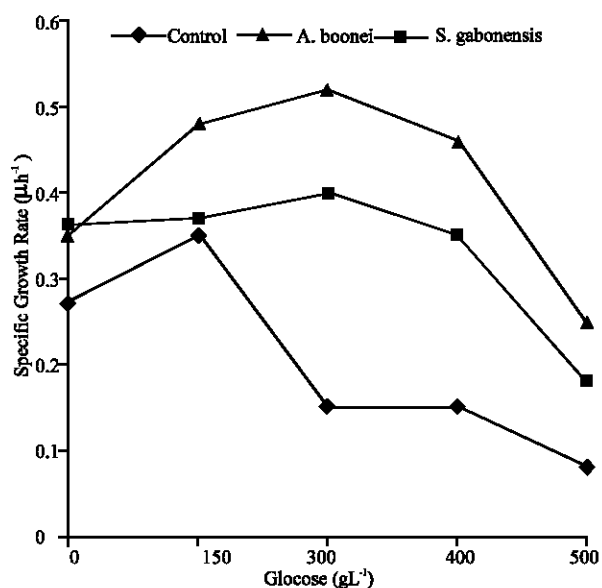

Fig. 1: Effect of plant product on osmotolerances of *S. Cerevisiae* Isolated from palm wine

Flocculation ability: The effect of plant products on the flocculation ability of *S. cerevisiae* isolated from palm wine (Fig. 2). Flocculation ability was monitored in terms of growth and aggregation of yeast cells. Actively growing yeasts are non- flocculent yeasts[20]. Results obtained indicated that both plant products reduced the flocculation ability of the yeast isolate. Untreated cultures had poor flocculation ability when compared with the standard flocculation brewer's yeast.

Flocculation is often exploited in the production of lager bear and wine. Flocs, which settle at the bottom of the fermenter by the end of primary fermentation, can easily be removed from the product, thereby making

Table 1: Morphological and Fermentation Characteristics of Yeast Isolated from the Palm Wine

Yeast Isolate	Colony Morphology	Cell Shape	Sugars														Probable Identity
			Fruc	Arab	Sor	Man	Suc	Xyl	Mal	Mel	Glu	Lac	Raff	Gal	Lys	Cat	
WY¹	Smooth Creamish	Round	AG	-	-	-	AG	-	AG	-	AG	-	A	AG	-	+	Saccharomyces cerevisiae
WY²	Elevated Creamish	Elongated	A	-	-	-	AG	A	A	-	AG	-	AG	-	+	+	Candida utilis
WY³	Smooth Creamish	Spherical	AG	-	-	-	AG	-	AG	-	AG	-	A	AG	-	+	*Saccharomyces cerevisiae*
WY⁴	Smooth Creamish	Oval	AG	-	-	-	AG	-	AG	AG	AG	-	AG	AG	-	+	*Saccharomyces uvarum*
WY⁵	Elevated	Spherical	AG	-	-	-	AG	-	AG	-	AG	-	A	AG	-	+	*Schizosaccharomyces pombe*

AG=Acid-gas production, A=Acid production, - = No fermentation, + =Fermentation, Fruc=Fructose, Arab=Arabinose, Sor=Sorbose, Man=Manitol, Suc=Sucrose, Xyl=Xylose, Mal=Maltose, Mel=Melibiose, Glu=Glucose, Lac=Lactose, Raff=Raffinose, Gal=Galactose, Lys=Lysine, Cat=Catalase.
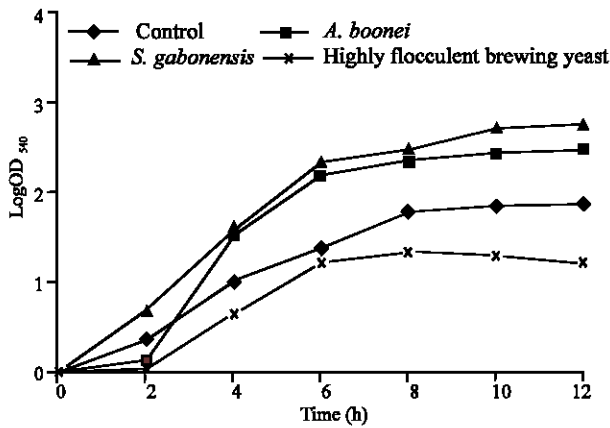

Fig. 2: Effect of plant products on the Growth kintics and flocculation pattern of S. Cereviasiae isolated from plam wine

Fig. 3: Effect of plant products on the Ethanol tolerance of S. Cerevisiae Isolated from plam wine

clarification rapid and efficient, with reduced handing of the wine[21].

Flocculation is a phenotypic property expressed by certain strains of yeast such as *S. cerevisiae*. It involves a sexual cellular aggregation when yeast cells adhere reversibly to one another, to form microscopic flocs which sediment out of suspension[22]. The genomic constitution, chemical and environmental factors affect the flocculation ability of yeast[23].

Most strains in a flocculent condition will disperse in the presence of sugar such as sucrose, maltose etc [20]. It is therefore possible that the sugars retained in the presence of the plant products may have acted as a dispersant for yeast cells.

Flocculation ability of yeast could be improved by the addition of $CaCl_2$ to the fermenting medium. Calcium chloride enhances the formation of flocs by linking cells through salt bridges between the calcium ions and the anionic polymers on the yeast surface[13]. Teunissen and Steensma[23] reported that calcium ions activate the production of flocculin (which are surface glycoproteins) capable of directly binding manoproteins of adjacent cells.
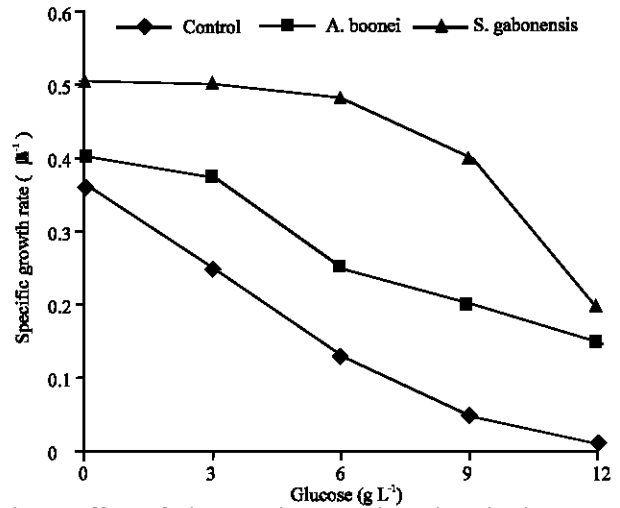
Table 2: Effect of plant products on alcohol production and invertase activity of *S.cerevisiae* isolated from palm wine

Sample	Alcohol Production (%ᵛ/ᵥ)	Invertase activity (UmL⁻¹)
Cultures without plant product (control)	6.42	16.40
Cultures containing A. boonei (0.50%)	11.53	15.20
Cultures containing S. gabonensis (0.625%)	12.07	2.40
LSD₀.₀₅	0.47	0.20

Ethanol production and tolerance: Result on the effect of *A. boonei* and *S. gabonensis* on alcohol production by the yeast is shown in Table 2. Plant products significantly (p<0.05) increased ethanol production by the yeasts. The culture containing *S. gabonensis* produced significantly higher (p<0.05) amount of ethanol (12.07%v/v) when compared with the culture containing *A. boonei* (11.53% v/v). Ethanol contents in cultures containing plant products were almost two times the ethanol (6.42% v/v) obtained from control sample. The observed alcohol values in the present study were lower than the maximum alcohol content (15% v/v) produced by a fusant of palm wine and brewer's yeast[15]. Figure 3 shows the effect of *A. boonei* and *S. gabonensis* on ethanol tolerance by the

yeast. The result showed that the plant products enhanced ethanol tolerance by the yeast. Ethanol tolerance by the yeast was higher in sample treated with *S. gabonensis* followed by the sample treated with *A. boonei* and then the control. The maximum growth rate of the yeast in both treated and untreated sample was recorded at 0% ethanol. There was a continuous decline of yeast growth in all samples, as the concentration of ethanol increased. The specific growth rate of the yeast (0.48) obtained from the culture containing *S. gabonensis* even in the presence of 6% v/v exogenous ethanol was higher than the specific growth rate of fusion yeast (0.32-0.42) reported by Ezeronye[15] . Kajiawara *et al.*,[24]and Boulton *et al.* [25] explained the decline in the fermentation ability of yeasts to be due to the combined action of ethanol on the yeast growth rate fermentation capacity, yeast cell viability associated with membrane lipid alteration. The ability of the yeasts to tolerate high concentration of ethanol therefore implies high fermentation capacity of the yeasts, enhanced yeast cell viability and growth in the presence of the plant products.

The ability of yeast to tolerate high concentration of ethanol is reported to be controlled by a large number of genes[22,26]. It is also related to cell membrane structures and functions[27]. It is possible that the plant products may have released into the fermenting medium, substances that can modify the yeast cell membrane structure and function thereby enhancing growth under high concentration of ethanol. High ethanol tolerance can enhance high ethanol yield and reduces the cost required for inoculum's development[28]. However, it is believed that alcohol productivity is not necessarily associated with high ethanol tolerance[29].

Osmotolerance: The effect of *A. boonei* and *S. gabanensis* on the osmotolerance of the yeast is shown in Fig 3. The results indicated that the plant products enhanced sugar tolerance by the yeast. It has been reported that high sugar concentration, up to 25% (v/v), represses the metabolic activity of yeast because of the osmotic effect of the sugar[30]. The maximum specific growth rate of the yeast was observed at 30% (v/v) glucose in the media treated with the plant products but declined thereafter. Crabtree effect appeared to have set in at glucose concentrations above 30% (v/v). High concentration of glucose has been reported to produce changes in enzyme components and influences cell wall structure[20]. The formation of mitochondria may be affected by high concentration of glucose. Subsequently, the respiratory chain will be affected. Under these conditions, enzymes of the Krebs cycle will have greatly lowered activities and sometimes are barely undetectable.

This will imply inability of the yeast to utilize sugar for energy production, hence poor growth results. The present study indicated that plant products enhanced osmotolerance as well as ethanol tolerance by the yeast. The combination of these two properties is an advantage when yeasts in being considered for ethanol production, thus highlighting the industrial potential of these two plant products. Sugar tolerance by yeast will allow higher concentration of sugar to be used for fermentation.

Invertase activity: The result in Table 2 showed that plant products reduced invertase activity of the yeast. The culture treated with *A. boonei* had significantly higher (p<0.05) invertase activity (15.20µ mL) than the culture treated with *S. gabonensis*. Invertase is an

extracellular enzyme found in the periplasm of the yeast cell wall, which hydrolyses sucrose into glucose and fructose. Sucrose is utilized by *S. cerevisiae* but is not transported into this organism; rather it is hydrolyzed in the periplasmic space by the secreted invertase[31]. High invertase activity enhances the capacity of yeasts to convert sucrose to ethanol. Harrison and Graham[32] reported that high invertase activity is required of yeasts for growth in molasses since the principal carbohydrate is sucrose. The results obtained from this study provide useful information necessary for further utilization of these plant products for industrial fermentation involving yeasts.

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