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Full Length Research Article

THE VIABILITY OF YEAST PRESERVATION ON PROTEASE PRODUCTION DURING SORGHUM WORT FERMENTATION

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ABSTRACT
The routine preservation of microorganisms promotes a complete depression of cell viability, stability and metabolic activity. In this study, *Saccharomyces uvarum* preserved under various conditions, was successfully reactivated within a relatively short time. The oils selected for preservation demonstrated higher cell viability and survival of yeast cells at cell count per mL of 2×10^6 cfu/mL Yeast cells preserved in 15 % glycerol in liquid-nitrogen all showed good survival rates and reactivation with 11×10^6 cfu/mL after 2 months. *Saccharomyces uvarum* was pitched in sorghum wort of two varieties (white & red) and fermentation was allowed to proceed. Results showed that protesse production was higher in red sorghum with yeast preserved in olive oil, mineral oil and corn oil. Production was optimal with isolate preserved under mineral oil and corn oil for 2, 4, 6 and 8 days during fermentation of white sorghum. The highest ethanol content of 27.8 % to 26.1 % was recorded with isolate preserved in mineral oil at room tempertaure for the white sorghum variety, with olive oil having the least yield of 15 %. Higher yield in both wort varieties was generated in the red sorghum wort fermented with yeast preserved in all oils at room temperature. Generally, there was better ethanol yield, cell viability and no apparent decrease in catalytic activity upon preservation , with yeast cells preserved at refrigerated temperature for the white sorghum and room temperature for the red sorghum. Preservation of yeasts cultures in mineral oil and corn oil provided better survival during storage period. Therefore, the use of some traditional methods for yeast culture preservation may be proposed.

Keywords: *Saccharomyces uvarum*, preservation, protease, viability and sorgum wort

INTRODUCTION
Microbial culture preservation offers convenience of storage that promotes a complete depression of all physiological activity (Nyanga *et al.,* 2012; Homolka, 2013). Mitigating changes in morphological, physiological and genetic traits of pure strains is the hallmark of preservation (Prakash, Nimonkar & Shouche, 2013). Microorganisms are exposed to a series of various stress factors in the environment, and keeping these factors under control during preservation, requires techniques indispensable in promoting growth suppression and metabolism (Cheong *et al.,* 2008). Yeast is a stable microbe inhabiting almost all environments on the earth and represent a powerful model system well suited for potential biocatalytic applications (Canbolat *et al.,* 2013). Storing yeasts on slant agar, with serial transfers is the basic aspect of yeast management. However, it creates undesirable morphological, physiological, genetic alterations, and increased risks of contamination (Prakash, Nimonkar & Shouche, 2013; Freitas *et al.,* 2011). Therefore, several preservation approaches that offers considerable promise in yeast management have been proposed (Homolka, 2013; Dahmen, Staub & Schwinn, 1983; Hubalek, & Kockova-Kratochvilova, 1981; Russell & Stewart, 1981). Low temperature preservation induces distinct responses among bacterial cells depending on the actual temperature (Obruca *et al.,* 2016). Cells become protected from contamination, ensuring viability with higher count, reduced production cost, ease of strain distribution, and short attenuation time (Pradelles *et al.,* 2009; Matoulková & Sigler, 2011; Ce, Mc & Se, 2015; Ale, Otero & Pasteris, 2015). Importantly, freezing of cells is accompanied by formation and propagation of intracellular ice, with concomitant detrimental effects (Fuller, 2004). However, when cryoprotective additives such as glycerol and DMSO are used, cell survival is maximal, and injury to the cellular structures by substantial dehydration is minimized (Mori *et al.,* 2012; Yu *et al.,* 2015; Watanabe *et al.,* 2018). Organic solvents are not considered suitable medium for preservation of yeast, as they strongly facilitate cell disruption (Linke *et al.,* 2010). Other yeast difficult to preserve, require modification of their sexual structures and auxotrophic requirements (Baeza *et al.,* 2009).
Preservation under a layer of high-quality mineral oil or liquid paraffin is considered appropriate for cultures that are amenable to freeze-drying. Retrieval of cultures stored in sterile water has also shown efficient growth (Smith, Mckay & Molina, 1994; Borman *et al.,* 2006; Ravimannan, 2016). Some cultures have also been successfully preserved in silica gel, soil, or sand, including filter paper (Delcán *et al.,* 2002; Cheong *et al.,* 2007). The viability and stability of preserved starters of yeast cultures improves organoleptic characteristics by producing intermediate alcohols, monoterpenes and volatile compounds (Clemente-Jimenez *et al.,* 2004; Jolly, Augustyn, & Pretorius, 2006; Hernndez-Orte *et al.,* 2008).Therefore, the use of yeast to produce enzymes offers certain advantages of moderate temperature growth, metabolic diversity, rapid cell growth, shorter fermentation cycles and easy adaptation to different cultivation conditions (Kato *et al.,* 2007).

Saccharomyces yeasts, can enhance the analytical composition and aroma profile of sorghum by production of secondary metabolites and secretion of enzymes when they interact with the sorghum compounds. The complete degradation of starch materials such as sorghum, is fundamental to industrial production of valuable products (Zyl, Bloom & Viktor, 2012). While cell viability remains the significant relevance of yeast fermentation in brewery, the characteristic physiological growth or enzymatic activities have been overlooked. Yeasts producing proteases may offer an alternative to bentonite treatment for removal of undesirable proteins in fermented products (Schlander *et al.,* 2017). This study concerns primarily *S. uvarum* yeast

applied in sorghum fermentation with the aim of evaluating to what extent the level of survival rates of preserved yeasts influenced yeast viability and retrieval as well as performance in protease production.

MATERIALS AND METHODS

Culture Conditions and Yeast Identification

Yeast cells were isolated by direct pour plating described by Harrigan and McCance (1966) from brewery flocs using Malt Extract agar and Yeast extract peptone dextrose agar (YEPDA) medium (supplemented with antibiotic). Yeast cells were carefully isolated after incubation for 3-5 days at 30 ^{o}C and routinely subcultured to obtain pure cultures and kept on Malt extract agar (MEA) slants at 4 ^{o}C. Fermentation test was conducted according to Wickerham (1951); Van der Walt and Yarrow (1984). Yeast isolates were tentatively identified by determining their pattern of fermentation and assimilation of glucose, sucrose, raffinose, galactose, maltose, lactose, and meliobiose according to Kurtzman and Fell (1998) and also carbon assimilation using API 32C (BioMereux SA. France) identification kit. Dilutions were made to provide cell concentration corresponding near to the scale of 5.0 MacFarland. Tubes in triplicate were incubated at 30 °C for 72 hours. The criterion for selection of yeast as fermentative was the turbidity of the medium, production and gas retention in Durham tube. Yeast cells were further examined by acid production from glucose, catalase and urease test.

Preservation conditions of yeast cells

Yeast suspensions at the OD of ~0.5 were prepared and added into a Minimal Broth [(5 g peptone, 3 g malt extract, 3 g yeast extract and glucose 5 % (w/v) per liter)] and preserved for 2 months at -10 ^{o}C. Sterile glycerol was added to 500 µL aliquots of late exponential phase yeast cultures to achieve 10 % and 15 % final concentration in a Nalgene cryogenic ampoules (Nalge Nunc Int. Parnorama Creek Rochester). The ampules were flame-sealed and quickly frozen by immersion in Liquid Nitrogen and stored for 2 months. Slants containing a 48 hr growth of Yeast cells were overlayed with mineral oil, castor oil, corn oil, olive oil and preserved for 2 weeks at room temperature (25 ^{o}C ± 2 ^{o}C) and refrigeration temperature (8 ^{o}C ± 2 ^{o}C). These oils were highly viscous, free of toxic or oxidized products and moisture.

Preparation of Sorghum Wort

The sorghum of the white variety (*Sorghum vulgare*) and red variety (*Sorghum bicolor*) were purchased from retailers at Bodija market in Ibadan Metropolis. Sorting of the sorghum grains to remove all extraneous matter was done. One kilogram of the grains were steeped under deionized water in a domestic plastic container for 20 hrs, drained and air rest for 4 hrs according to modify methods of Agu & Palmer, (1996). About 0.1 % Formaldehyde was added to the steep water to reduce microbial load. Dried grains were spread on pre-wetted jute bags and covered with pre-wetted jute bag and allowed to sprout at room for 96 hours. The grains were turned at 24 hour intervals to avoid excessive malting and distilled water sprinkled. Kilning was done to obtain crispy sorghum malt by drying at 65 ^{o}C in a hot air oven (Gallenhamp moisture extraction oven model ov-335) for 16 hrs until the rootlets were friable. The dried malted sorghum grains were milled with a sample miller (CytotechTM 1093 sample mill Sweden) and used for wort preparation. Modified decantation mashing procedure of Palmer, (1989) was employed for the wort preparation. Powdered malt (50g) was used for the single decoction mashing process and the flour extracted with 300 mL of distilled at a temperature of 45 ^{o}C in a mash bath for 30 min. The enzymatically active wort was decanted and the starchy-grist residue was heated to 100 ^{o}C to gelatinize the starch. The mash was then cooled and the decanted wort was re-added to achieve a conversion temperature of 65 ^{o}C (Igyor, Ogbonna & Palmer, 2001; Etokakpan & Palmer, 1990). The volume was adjusted to 400 mL and maintained at 65 ^{o}C for 1 hr. The liquid extract (wort) was then separated from the spent grain particles with a clean muslin cloth and preserved at 4 ^{o}C. The Malting Loss was ascertained as described by described by Dewar, Taylor & Berjak, (1997), and the alcohol content determined according to AOAC (1990).

Inoculum size determination and fermentation of the sorghum wort.

The yeast cells were harvested by centrifugation after preservation and suspended in 10 mL of sterile deionized water. The cell suspension (1 mL) was introduced into the counting chamber of a Neubauer haemocytometer (0.1 mn depth). Viability was assessed using the methylene blue staining method (Lekkas *et al.,* 2007). The charged counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. The number of cells within the squares (0.1 mm^3) in each of the 3*3 grid (i.e outside the central tripple ruled area) were counted and the number of cells counted and multiplied by 104 to get the number of cells per mL. If the cell suspension was diluted to 10 fold, the final count will be multiplied by the dilution factor. Fermentation was carried out by inoculating 20 mL of sorghum wort dispensed into srew capped bottles with suspension of the test organism at a pitching rate of 2.0×10^6 cells/mL. Inoculation was done under strict aseptic conditions and the scewed capped bottles incubated at 30 ^{o}C for 7 days.

Ammonium sulphate precipitation.

The crude enzyme filtrate were pooled together and partially purified using the Ammonium Sulphate precipitation method at 0-40 % and 40-80 % saturation, respectively. The mixture of each saturation was stirred continously until the Ammonium Sulphate completely dissolved. The mixture was kept at 4 ^{o}C for 18 hrs after which it was centrifuged at 2500 rpm for 10 min. The precipitate was suspended to the initial volume of crude filtrate with 0.002 M sodium phosphate buffer pH 6.9 and used for further analysis.

Protease assay

This was done using the modified method of Kunitz (1947). About 1 % casein (BDH) was prepared in 0.05 M Citrate phosphate buffer (pH 7.0) and heat denatured at 100 ^{o}C in a water bath for 15 mins. The solution was allowed to equilibrate at 35 ^{o}C before use. To 1 mL casein solution in a tube was added 1 mL of the enzyme preparation mixed and incubated at 35 ^{o}C for 1hr in a water bath. The reaction was terminated by adding 3 mL of cold (2 ^{o}C) 10 % TCA. The control tube contained only casein solution during incubation at 35 ^{o}C into which TCA was added before the addition of enzyme preparation. The undigested protein were allowed to precipitate by standing the tubes at 2 ^{o}C for 1 hr in a refrigarator. The reaction mixtures were then centrifuged at 10,000 rpm at 4 ^{o}C for 5 mins. The optical density readings of the

carefully decanted supernatant fluid were then measured with a Perkin Elmer UV/Visible Spectrophotometer at 660nm wavelenght against a blank containing the control.One protease unit is defined as the amount of enzyme that released 1µg of casein protein per mL per minute under the above stated assay conditions.

Statistical analysis

The general linear model (GLM) of SPSS statistical package (Version 17) was used for the statistical analysis of results. Data obtained were examined statistically for variation among treatment conditions using Duncan Multiple Range Tests (one way ANOVA). The level of significant was $p<0.05$, except if otherwise stated.

RESULTS

Yeast Identification and carbon assimilation

A total of sixteen yeasts cells were randomly isolated from 'breweries flocs' collected from Nigerian Breweries in Ibadan. Isolates were narrowed down to eight after differentiation on the basis of their cultural, morphological, microscopic observation and pigmentation. Isolates were identified as *Saccharomyces uvarum* according to the Compendium of Lower Fungi Isolation, Classification and Maintenance of Yeast (Van der Walt and Yarrow 1984). The purified colonies were described in relation to morphological features such as edge, color, texture and shine (Kreger-Van Rij 1984). All isolates appeared oval and ellipsoidal and growth was accompanied by the formation of creamy/whitish pigment. Growth on morphology agar showed a spread pattern of growth of isolates with smooth ages and mucoid texture. Microscopic observation showed small budding vegetative yeast cells.

Yeast cells showed a rapid fermentation of sugars with the production of gas with the exception of raffinose and lactose **(Table 1).** Fermentation of melibiose (an important sugar in *S. uvarum* identification) was rapid within 18 hrs. of inoculation of the fermentation broth. The growth characteristics and ability of these isolates to ferment melibiose confirmed the yeast cells as *Saccharomyces uvarum.* This has been previously documented by Bamforth (2006), according to whom the basic difference between *S. uvarum* and other brewing yeasts strains is the ability to ferment the sugar melibiose.

Table 1: Physiological and Biochemical Properties of the yeast Isolates

Isolates	Growth at 50% Glucose	Growth at 37°C	Growth at 25°C	Urease	Gelatin Liquefaction	Growth in $CaCO_3$	Glu	Mal	Suc	Raf	Gal	Mel	Lac	Probable species
M1	+	+	+	-	-	-	+	+	+	-	+	+	-	*Saccharomyces. uvarum*
M2	+	+	+	-	-	-	+	W+	-	-	+	+	-	*Saccharomyces spp.*
M3	+	+	+	-	-	+	+	+	+	-	+	+	-	*Saccharomyces uvarum*
M4	+	+	+	-	-	+	+	+	+	-	+	+	-	*Saccharomyces uvarum*
M5	+	+	+	-	-	+	+	+	+	-	W+	+	-	*Saccharomyces spp.*
M6	+	+	+	-	-	+	+	+	+	-	+	+	-	*Saccharomyces uvarum*
M7	+	+	+	-	-	+	+	+	+	-	+	+	-	*Saccharomyces uvarum*
M8	+	+	+	-	-	+	+	+	+	-	+	+	-	*Saccharomyces uvarum*

Key: (+) = positive reaction, (−) = negative reaction. (Glu)-Glucose, (Mal)-Maltose, (Suc)-Sucrose, (Raf)-Raffinose, (Gal)-galactose, (Mel)-melibiose, (Lac)-Lactose

Four isolates were further differentiated by the carbon assimilation tests using the API 32C. This allowed the screening of cultures based on the ability to assimilate different carbon compounds as carbon source **(Table 2)**. Results were interpreted based on turbidity of inoculated medium when compared to an uninoculated control medium).

Table 2: Carbon Assimilation in Yeast Sugar Fermentation

Isolates	Gal	Suc	Gluc	Sor	Mal	Raf	Cel	Rham	Treh	Melz	Lac	Arab	Rib	Xyl	Mel	2-Keto	Probable Species
M2	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	*S. uvarum*
M4	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	*S. uvarum*
M7	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	*S. uvarum*
M8	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	*S. uvarum*

Key: Gal (Galactose), Suc (Sucrose), Gluc (Glucose), Sor (Sorbose), Mal (Maltose), Raf (Raffinose), Cel (Cellibiose), Rham (Rhamnose) Treh

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(Trehalose), Melz (Melezitose), Lac (Lactose), Arab (Arabinose), Rib (Ribose), Xyl (Xylose), Mel (Melibiose), 2-Keto (2-Ketoglutarate)

The results of the characteristic properties of the sorghum wort varieties prior to fermentation **(Table 3)** shows the pH to be below neutral assuming an acidic state. The fermentative metabolism of yeast cells is better mediated in slightly acidic than in alkaline conditions. Therefore, the pH of the wort concentration is considered suitable for the fermentation by the yeast. Consequently, both wort concentration maintained the same specific gravity with more malting loss recorded for red sorghum wort variety. The pitching rate was maintained at the initial state of inoculation to give a uniform yeast growth succession.

Table 3: Characteristics of the sorghum wort prior to fermentation

Characteristics	Sorghum white (SW)	Sorghum red (SR)
Specific gravity	1.043	1.045
Pitching rate	2.0×10^6	2.0×10^6
pH	5.1	5.2
Malting loss	20.08%	29.23%

Viability of *S. uvarum* yeast cells after preservation in different oils

Apparently the cell viability of *S. uvarum* preserved in different oils was observed to have been remarkably uniform after each preservation periods and conditions **(Figure 1).** The viability is indicated by live/dead staining and there was a progressive increase in number of yeast cells and a gradual decline during the 10 days of preservation of *S.uvarum* in all the oils under different condiitons. This suggest the metabolic viability of the yeast cells to be unaffected by the preservatives as well as the conditions. More so, it highlights the characteristic trait of *S. uvarum* of a rapid growth and large cell mass. It was however, observed that cell counts was higher when the yeast cells were preserved under refrigeration condition. As expected the maximum yeast cell count was higher in the case of yeast cells preserved in 10 % and 15 % glycerol in liquid nirogen with 7.9×10^6 CFU/mL and 11×10^6 CFU/mL after 2 months, which demonstrates the lyophilization as the best method for manatnaing yeast viability. The lyophilization lowers the metabolic rate of the organism which further reduces the chances of occurrence of cell death.

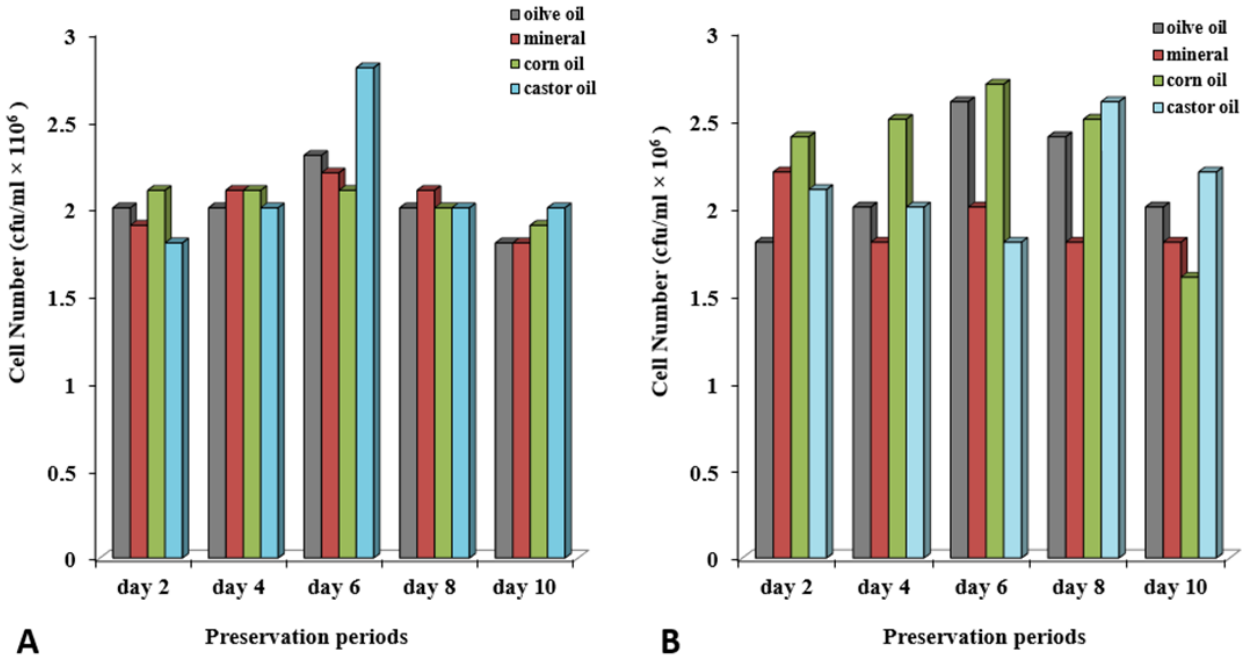

Figure 1: Neuber haemocytoetry cell viability of *S. uvarum* preserved under different oils at (A) room temperature, and (B) refrigeration Temperature

Preservation Period of *S. Uvarum* on its (%) Alcohol Yield

The ethanol content of the white sorghum wort at 7 days fermentation increased sharply when compared with unfermented wort **(Figure 2 and Figure 3).** There was increased yield with isolate preserved in mineral oil, corn oil and castor oil for 2, 4, and 6 days at room temperature. The highest yield was recorded with isolate preserved in mineral oil for 4 days (27.8%) and 6 days (26.1%). The lowest yield was recorded with wort fermented with isolate preserved in olive oil for 6 days (14.8%), 8 days (13.8%) and 10 days (13.4%) at room temperature. Yeast preserved in castor oil at refrigeration temperature showed less ethanol yield with an initial increase with isolate preserved for 2 days and a sharp decline with isolate preserved for 4, 6, 8, and 10 days. Duncan Multiple Range Analysis of the means of ethanol yield at $P< 0.05$ showed that there was no significant difference between the preservation conditions on ethanol yield by isolate preserved in mineral oil. White sorghum wort fermented with isolate preserved in mineral oil at refrigeration temperature performed better.

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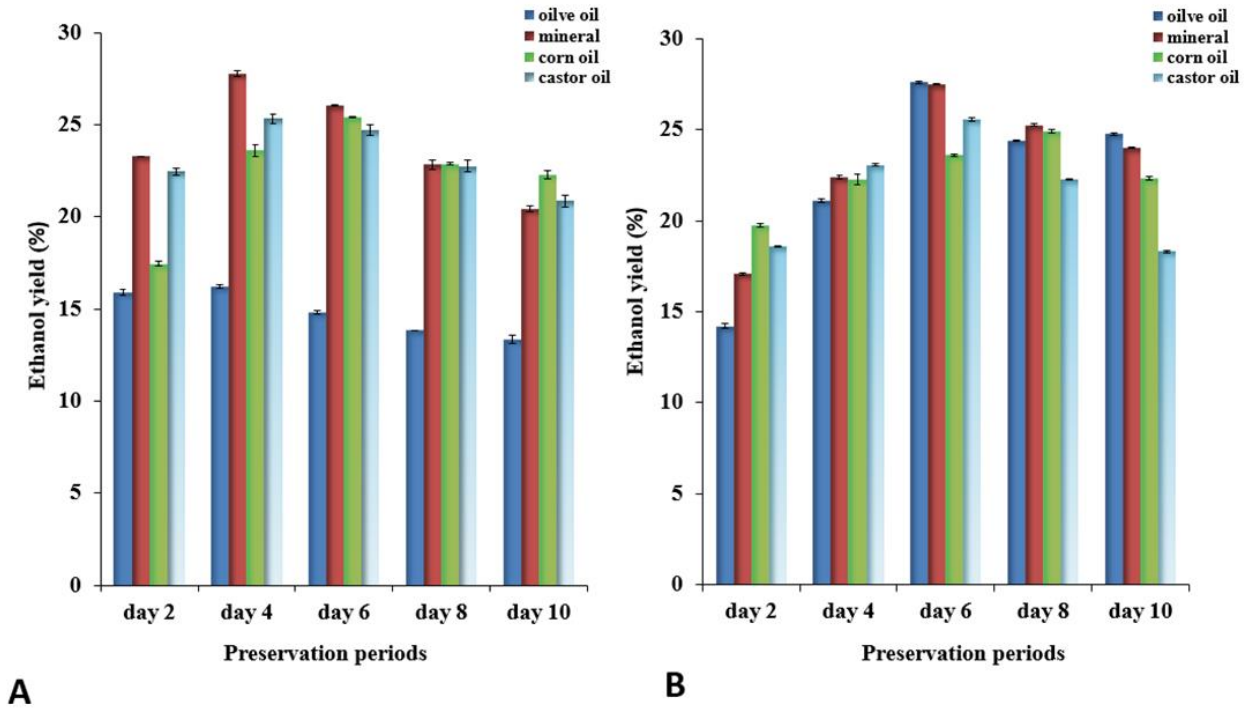

Figure 2: Ethanol yield of *S. uvarum* fermentation of white sorghum after preservation under different oils at (A) room tempertaure and (B) refrigertaion temperature

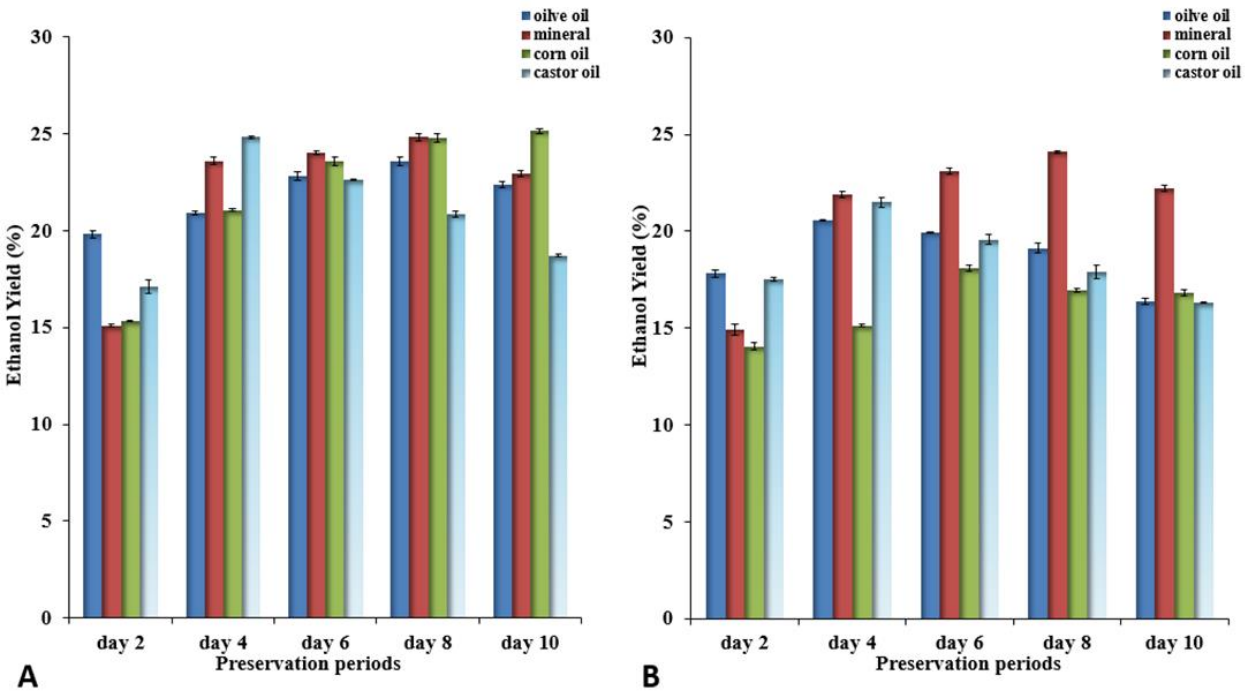

Figure 3: Ethanol yield of *S. uvarum* fermentation of red sorghum after preservation under different oils at (A) room tempertaure and (B) refrigertaion temperature

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The ethanol yield in fermented red sorghum wort showed a decline with yeast preserved at refrigeration temperature (Figure 2). There was increased yield with isolate preserved in mineral oil (4, 6 & 8 days) and corn oil (6, 8 & 10 days) at room temperature. The highest yield recorded was corn oil for 8 days (24.8 % v/v) and 10 days (25.1 % v/v) followed by isolate preserved in mineral oil for 6 days (24.0% v/v) and 8 days (24.8%). At refrigeration temperature yeast preserved in corn oil for 2 days and 4 days showed lower ethanol yield of 14.1 % (v/v) & 15.2 % (v/v) respectively. Isolate preserved in mineral oil at refrigeration temperature for 6 days and 8 days showed the highest yield of 23.1 % (v/v) & 24.1 % (v/v), respectively.

Protease activity of preserve *S. uvarum* during fermentation of white and red sorghum wort

Protease production potential of the preserved and recovered yeast cells in white sorghum fermentation are as shown in **Figure 4.** The yeast cells preserved in olive oil at room temperature had increasing activities with a slight drop in activity on the 10th day of fermentation. The protease activity was also higher with yeast preserved in mineral oil at room temperature with a peak of 2 × 10^3 Units/mL on day 6 of the fermentation. Yeast preserved and recovered from all oils at room temperature all showed significant increase in protease production from day 2 to day 10 of fermentation but it was however, observed that all the yeast preserved in the different oils showed similar pattern of decline from day 8 of fermentation in the protease production. Protease production declined faster on day 10 of fermentation with yeast preserved in castor oil. Observations recorded for yeast preserved in different oils at refrigeration temperature showed a markedly significant performance in the protease production. Protease production was in an increasing amount with yeast preserved in mineral oil during the wort fermentation. Although higher protease production was observed for the same mineral oil on day 6 with yeast preserved at room temperature, the production was higher and steady with yeast preserved at refrigeration temperature from day 2-8 of the fermentation. Yeast preserved in castor and corn oil manifested slow protease production with day 2 and day 4 of fermentation. Olive oil preserved yeast had the sharp decline in protease production but had better protease production in the early days of fermentation. Generally, there was a clear-cut difference in the behaviour of the yeast preserved under the two different conditions of temperature. Yeast preserved in all oils at room temperature had stability and viability in protease production during the fermentation periods better that yeast preserved under refrigeration conditions.

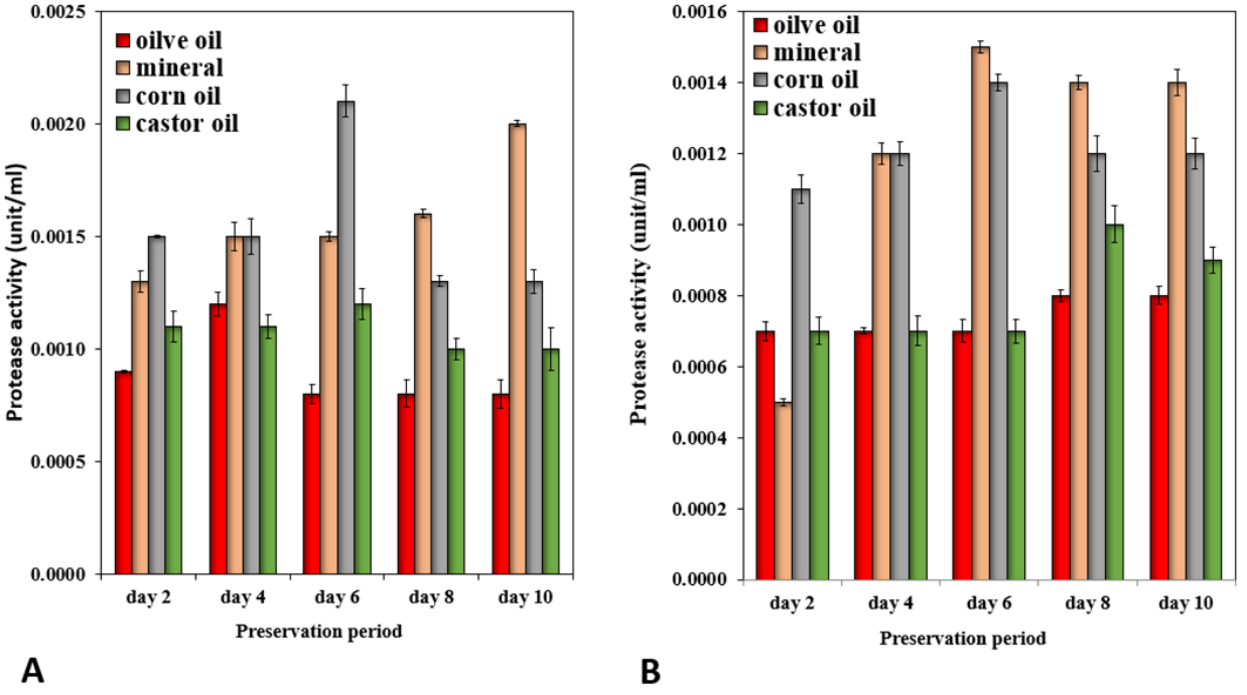

Figure 4: Influence of preserved *S. uvarum* yeast under different oils at (A) room temperture (B) refrigeration temperature, on protease production during fermentation of white sorghum wort

Protease production as observed during fermentation of red sorhum wort with yeast cells preserved in different oils under room and refrigerated conditions are shown in **Figure 5**. There was a markedly observed difference in the performance of the yeast, particularly during the red sorghum wort fermentation with yeast preserved under the room temperature. Yeast preserved in corn oil showed higher potential of protease production to about 2.5 × 10^3 Unit/mL of activity. Similar observations can be drawn from the protease activity of yeast preserved in mineral oil at room temperature which showed a progressive amount of activity. Production of protease was poor for yeast preserved in olive oil at room temperature. In a sharp contrast, mineral and corn oil

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sustained the metabolic performance of the yeast during preservation under refrigerated conditons. This is evident in the higher activity of protease recorded throughout the duration of the fermentation. It was however, observed that yeast preserved in olive oil and mineral oil under refrigeration, performed poorly as indictaed by the lower protease activity during the red sorghum wort fermnetation.

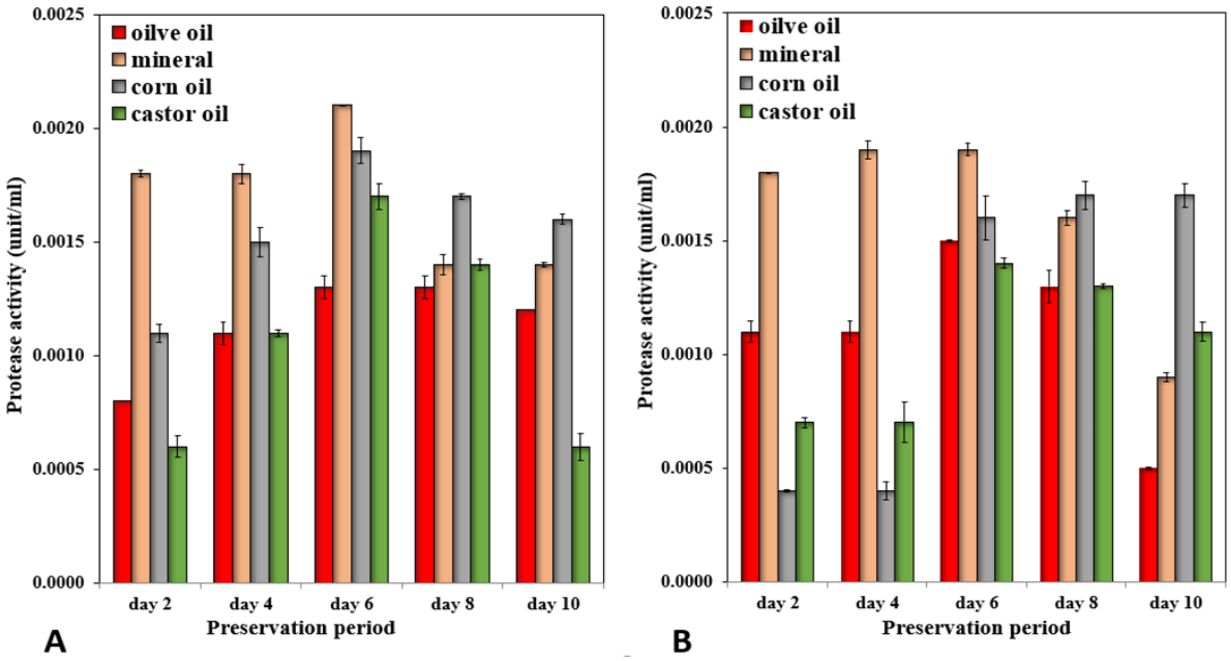

Figure 5: Influence of preserved *S. uvarum* yeast under different oils at (A) room temperture (B) refrigeration temperature, on protease production during fermentation of red sorghum wort

Preservation of *S. uvarum* in liquid nitrogen and 5 % glucose on protease production

The preservation of yeast for two months in glucose sugar and liquid nitrogen was conducted with the yeast mixed in 10-15 % glycerol. Results as shown in **Figure 6,** indicated there was no diminished effect on the proteolytic activity of *S. uvarum* following fermentation of both white and red sorghum wort. The better performance of the yeast fermentation in both white and red sorghum wort highlights the importance of the sorghum variety as a preferred choice for most brewers, and more importantly the significant role cryopreservation still plays in yeast viability and stability.

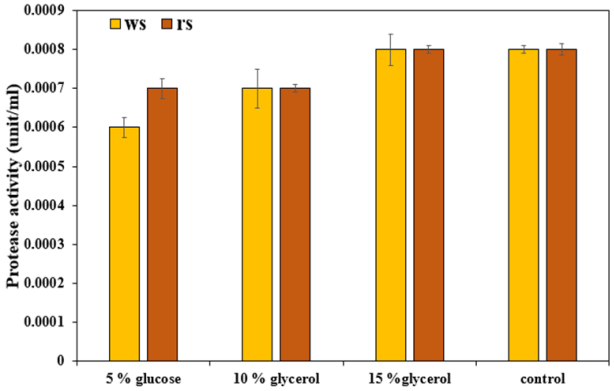

Figure 6: Influece of preserved yeast under 5 % glucose and 10-15 % glycerol (Liquid nitrogen) respectively, on protease production during white and red sorghum wort fermentation.

DISCUSSION

The fundamental value of these methods of yeast culture maintenance depends upon the retention by the yeast of all its properties unchanged, increasing the reliability of using the same batch of inoculum for a very long period of time (Cheong *et al.*, 2008; Prakash *et al.*, 2013; Suga, Isobe, & Hatakeyama, 2000; Hubálek & Kocková-Kratochvílová, 1982). In this study the preservation of yeast in diferrent oils at different conditions didn`t show any marked defects on the performance of the yeast. Generally, the viability of yeast cultures following long-term storage can range from 5 to 97 % when compared with viability before storage (Hubálek & Kocková-Kratochvílová, 1982). Although preservation conditions sometimes affects yeast viability, the viability of yeast encourages the fermentation process as growing cells have been said to produce alcohol several times faster than non-growing cells (Ingledew & Patterson, 1999). The viability of yeast cell count can only be supported by the level of fermentable sugars and other nutrients provided in the medium by the hydrolytic activity of the enzymes during wort preparation and fermentation.

The yeast cell viability was remarkably higher in corn and mineral oil; these oils preserved the isolate by limiting the access of oxygen, diminishing the culture growth and metabolism, i.e. hypobiosis is achieved. The use of oils in preservation prevents accidental selection and spontaneous mutations that would most likely cause changes in the microbial phenotype and genotype (Donev 2001). Selection of oils for microbial preservation is very

critical to the viability of the yeast cells. Although not observed during the course of this study, morphological changes in some yeasts and microscopic fungi can occur in the long-term storage using oil (Donev, Savova, & Kujumdzieva, 1995). This is because it was long established that thermal processing of oils especially during sterilization can results in moisture development leading to formation of side toxic products in the oils (Kuyumdzhieva-Savova *et al.,* 1985) had reported.

During the preservation of *S. uvarum* in liquid nitrogen (LN), the glycerol acted as a cryoprotector providing protective effect against the unfavourable environmental conditions and cryogenic influences found during preservation in liquid nitrogen. Furthermore, many yeasts can grow on glycerol as a sole carbon source under aerobic conditions, but glycerol is a non-fermentable carbon source for many yeast species, including *Saccharomyces spp*. Glycerol functions as a compatible solute in osmoregulation in osmotolerant yeasts that are capable of growing in high sugar or salt environments. To serve as a carbon source, glycerol after internalization has to be converted by glycerol kinase to glycerol-3-phosphate, which is then transformed into DAH phosphate by glycero-l-3-phosphate dehydrogenase; a substrate in gluconeogenesis (Bidlingmaier *et al.,* 2001; Bouquin *et al.,* 2000).

The impact of pitching rate on fermentation and beer quality parameters has not been studied systematically. Verbelen *et al.,* (2008) found that fermentation rate increased extensively and the net yeast growth was lowered with increasing pitching rate even though the viability and vitality of the yeast population were not significantly affected. However, there was no significant decrease in cell viability for all preservation conditions employed in this study. Yeast cells manifested a steady pitching cell viability particularly in the oil preservation. The maximum viable cell numbers increase with increase in the rate of fermentation in order of increasing pitching rate. Precise pitching of yeast ensures a minimum lag period and rapid fermentation, resulting in the drop of wort pH quickly , hence decreasing possible bacterial growth.

The primary goal for industrial yeast is to convert sugar to ethanol and carbon dioxide and still maintain high ethanol tolerance. Rapid ethanol production during fermentation by yeast is as a result of the shortening of the adaptation phase prior to the conditioning phase, while the gradual increase in ethanol after a period of time can be attributed to the slow progress of death phase (Palmer & Agu, 1999). The different wort samples fermented with the preserved isolate showed almost no difference concerning the apparent quantity and intrinsic alcohol content produced. This observation was also established by Yonkova *et al.,* (2007), when estimating the quality of product by measuring the alcohol produced. Osho, (2005), also observed that ethanol tolerant yeast tend to be sugar tolerant. This is also true for *S. uvarum* which has the ability to ferment sugars at a high rate and has a high tolerance to alcohol (Rosini *et al.,* 1982).Yet at certain concentrations, ethanol is very toxic to the yeast cell. The inhibitory action of ethanol produced in the course of fermentation or added externally is complex. Ethanol production has been reported to be slightly faster during fermentation in white sorghum than red sorghum (Demuyakor & Ohta, 1993).

Sorghum wort were observed not to vary significantly in their performance when considering their pre and post fermentation characteristics. However, the white sorghum charateristics preference over the red sorghum as reported by Pozo-Insfran *et al.,* (2012) and Okungbowa, Obeta, & Ezeogu, (2002) is in the use of different biosynthesis during wort preparation or the ability to produce more enzymes during malting (Dicko *et al.,* 2006). It was observed in this study that higher enzyme activity was demonstrated in wort of white sorghum after fermentation.

Sorghum varieteis in their grain contained the enzymes proteases, amylases and glucanases. The activities of these enzymes are mostly lost during wort preparation. However, according to Bajomo & Young, (1993) much research on sorghum has centred mainly around its malting and mashing potential. The protease enzymes hydrolyse the sorghum starch and carbohydrates at the malting and mashing stage, but brewers utilize unacceptably high levels of commercial enzymes to assist with the hydrolysis. This consequently increases the content of fermentable carbohydrates and proteins hence reducing dextrins.Yonkova et al., (2007) also suggested the use of industrial enzymes mixtures to balance the composition of the wort. It is reported that brewing with sorghum malt is associated with some shortcomings including its proteolytic acitivity deficiencies as well as reduced fermenatbility of its worts by brewing yeast (Palmer et al., 2002). From this statement and the findings in this work, it holds then that yeast ability to produce this enzymes extracellulaly would complement these shortcomings. Sorghum malt proteases play important roles in seed structure modification during germination and the generation of free amino acids during lager beer brewing. This study showed an apparent proteolytic activity of the yeast during wort fermentation and dynamic changes in the activity profile. This is consistent with Lekkas *et al.,* (2007) remarks that, protease account for the complex mixture of amino acids, peptides, nucleic acids and other constituents of wort, and the changes in extracellular protease activity reveals the dynamic environment that develops during fermentation. The findings in this work expresses a contrasting view with that of Ingledew (2000), who stablished that brewer`s yeast demonstrate no appreciable proteolytic activity in wort. Although, they do produce enzymes inside the cell, yeast does not excrete proteases and as a result there is no protein degradation taking place in wort during fermentation. On the other hand, Lekkas *et al.,* (2007) proposed that, extracellular yeast proteolytic enzymes are responsible for the degradation of lager wort peptides into smaller ones providing yeast cells with more available assimilable nitrogen sources.

Conclusion

The use of some traditional methods for yeast culture preservation as demonstarted in this study may be proposed or advocated as they appear to be more promising. The study confirms that the yeast maintains its viability as well as the ability to express enzymatic capacity without signifcant dimishing effect of the preservation conditions. These preservation methods also showed satisfactory performance on the yeast, stability in growth and yeast viability with minimal detrimental effects indicating that the physiological condition of the yeast is not impaired. This study also offers information on the proteolytic potential of *S.uvarum*, which can be a feasible option in reducing cost when compared to the use of commercial enzymes at higher cost

REFERENCES

Abadias, M., Teixido, N., Usall, J., Benabarre, A., Vinas, I. (2001). Viability, efficacy, and storage stability of freeze-dried biocontrol agent Candida sake using different protective and rehydration media. *Journal of Food Protection*, *64*(6), 856–861.

Agu, R. C., & Palmer, G. H. (1996). Enzymic breakdown of endosperm proteins of sorghum at different malting temperatures. *Journal of the Institute of Brewing*, *102,* 415-418.

Ale, C. E., Otero, M. C., & Pasteris., S. E. (2015). Freeze-drying of wine yeasts and *Oenococcus oeni* and selection of the inoculation conditions after storage. *Journal of Bioprocessing & Biotechniques*, *05*(08).

Baeza, M., Retamales, P., Sepúlveda, D., Lodato, P., Jiménez, A., & Cifuentes, V. (2009). Isolation, characterization and long term preservation of mutant strains of Xanthophyllomyces dendrorhous. *Journal of Basic Microbiology*, *49*(2), 135–141.

Bajomo, M. F., & Young, T. W. (1993). The properties, composition and fermentabilities of worts made from 100% raw sorghum and commercial enzymes. *Journal of the Institute of Brewing*, *98*(6), 515–523.

Bidlingmaier, S., Weiss, E. L., Seidel, C., Drubin, D. G., & Snyder, M. (2001). The Cbk1p Pathway Is Important for Polarized Cell Growth and Cell Separation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 21(7), 2449–2462.

Borman, A. M., Szekely, A., Campbell, C. K., & Johnson, E. M. (2006). Evaluation of the viability of pathogenic filamentous fungi after prolonged storage in sterile water and review of recent published studies on storage methods. *Mycopathologia*, *161*, 361–368.

Bouquin, N., Barral, Y., Courbeyrette, R., Blondel, M., Snyder, M., & Mann, C. (2000). Regulation of cytokinesis by the Elm1 protein kinase in *Saccharomyces cerevisiae*. *Journal of Cell Science*, *113 (Pt 8*, 1435–1445.

Canbolat, M. F., Gera, N., Tang, C., Monian, B., Rao, B. M., Pourdeyhimi, B., & Khan, S. A. (2013). Preservation of Cell Viability and Protein Conformation on Immobilization within Nanofibers via Electrospinning Functionalized Yeast. *ACS Applied Materials & Interfaces*, *5*(19), 9349–9354.

Cheong, C., Wackerbauer, K., Beckmann, M., Jang, K., & Kang, S. A. (2008). Effect of cultivation conditions on trehalose content and viability of brewing yeast following preservation via filter paper or lyophilization methods. *Biotechnology and Bioprocess Engineering*, *13*, 690–696.

Cheong, C., Wackerbauer, K., Beckmann, M., & Kang, S. A. (2007). Influence of preserved brewing yeast strains on fermentation behavior and flocculation capacity. *Nutrition Research and Practice*, *1*(4), 260–265.

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Heras-Vázquez, F. J. Las, & Rodríguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiology*, *21*(2), 149–155.

Dahmen, H., Staub, T., & Schwinn, F. J. (1983). Technique for Long-Term Preservation of Phytopathogenic Fungi in Liquid Nitrogen. *Techniques*, *73*(2), 241–246.

Demuyakor, B., & Ohta, Y. (1993). Characteristics of single and mixed culture fermentation of pito beer. *Journal of the Science of Food and Agriculture*, *62*(4), 401–408.

Dewar, J., Taylor, J. R. N., & Berjak, P. (1997). Determination of Improved Steeping Conditions for Sorghum Malting. *Journal of Cereal Science*, *26*, 129–136.

Dicko, M. H., Gruppen, H., Traoré, A. S., Voragen, A. G. J. & van Berkel, W. J. H. (2006). Sorghum grain as human food in Africa: relevance of starch content and amylase activities. *African Journal of Biotechnology*, *5*(5), 384–395.

Donev, T., Savova, I., & Kujumdzieva, A. (1995). Influence of the cultivation conditions on yeast strains survival after lyophilization. *Journal of Culture Collections*, *1*(January 1995), 38–41.

Etokakpan, O. U., & Palmer, G. H. (1990). Comparative studies of the development of endosperm-degrading enzymes in malting sorghum and barley. *World Journal of Microbiology and Biotechnology*, (1975), 408–417.

Freitas, R. S., Dantas, K. C., Pereira, C. N., Levi, J. E., Eduardo, J., & Martins, C. (2011). Preservation methods of fungi in 35 years old stock culture storages : A comparative study. *African Journal of Microbiology Research*, *5*(5), 555–561.

Fuller, B. J. (2004). Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryo Letters*, *25*(6), 375–388.

Homolka, L. (2013). Methods of Cryopreservation in fungi. *Current Methods in Fungal Biology*, 9–17.

Hubálek, Z., & Kocková-Kratochvílová, A. (1982). Long-term preservation of yeast cultures in liquid nitrogen. *Folia Microbiologica*, *27*(4), 242–244.

Igyor, M. A., Ogbonna, A. C., & Palmer, G. H. (2001). Effect of malting temperature and mashing methods on sorghum wort composition and beer flavour. *Process Biochemistry*, *36*, 1039–1044.

Ingledew, W. M., & Patterson, C. A. (1999). Effect of Nitrogen Source and Concentration on the Uptake of Peptides by a Lager Yeast in Continuous Culture. *Journal of the American Society of Brewing Chemists*, *57*(1), 9–17.

Delcán, J., Moyano, C., Raposo, R., & Melgarejo, P. (2002). Storage of *Botrytis cinerea* using different methods. *Journal of Plant Pathology*, *84*(1), 3–9.

Hernandezorte, P., Cersosimo, M., Loscos, N., Cacho, J., Garciamoruno, E., & Ferreira, V. (2008). The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chemistry*, *107*(3), 1064–1077.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2006). The Role and Use of Non- Saccharomyces Yeasts in Wine Production. *S. Afr. J. Enol. Vitic*, *27*(1), 15–39.

Kato, M., Fuchimoto, J., Tanino, T., Kondo, A., Fukuda, H., & Ueda, M. (2007). Preparation of a whole-cell biocatalyst of mutated *Candida antarctica* lipase B (mCALB) by a yeast molecular display system and its practical properties. *Applied Microbiology and Biotechnology*, *75*(3), 549–555.

Kuyumdzhieva-Savova, A., Savov, V. A., Genova, L. K., & Peikova, S. P. (1985). Superoxide dismutase in methylotrophic yeasts. *FEMS Microbiology Letters*, *27*(1), 103–105.

Lekkas, C., Stewart, G. G., Hill, A. E., Taidi, B., & Hodgson, J. (2007). Elucidation of the role of nitrogenous wort components in yeast fermentation. *Journal of the Institute of Brewing*, *113*(1), 3–8.

Linke, B., Schröder, K., Arter, J., Gasperazzo, T., Woehlecke, H., & Ehwald, R. (2010). Extraction of nucleic acids from yeast cells and plant tissues using ethanol as medium for sample preservation and cell disruption. *BioTechniques*, *49*(3), 655–657.

Mori, S., Choi, J., Devireddy, R. V., & Bischof, J. C. (2012). Calorimetric measurement of water transport and intracellular ice formation during freezing in cell

suspensions. *Cryobiology*, *65*(3), 242–255.

Nyanga, L. K., Nout, M. J. R., Smid, E. J., Boekhout, T., & Zwietering, M. H. (2012). Yeasts preservation: alternatives for lyophilisation. *World Journal of Microbiology and Biotechnology*, *28*(11), 3239–3244.

Obruca, S., Sedlacek, P., Krzyzanek, V., Mravec, F., Hrubanova, K., Samek, O., … Marova, I. (2016). Accumulation of Poly(3-hydroxybutyrate) Helps Bacterial Cells to Survive Freezing. *PLOS ONE*, *11*(6), e0157778.

Okungbowa, J., Obeta, J. A. N., & Ezeogu, L. I. (2002). Sorghum β-Amylase Production: Relationship With Grain Cultivar, Steep Regime, Steep Liquor Composition and Kilning Temperature. *Journal of the Institute of Brewing*, *108*(3), 362–370.

Osho, A. (2005). Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice. *African Journal of Biotechnology*, *4*(7), 660–662.

Palmer, G. H., & Agu, R. C. (1999). Effect of Mashing Temperatures and Endo-β-Glucanase on β-Glucan Content of Malt Worts. *Journal of the Institute of Brewing*, 105(4), 233–236.

Palmer, J. M., Sandalio, L. M., Corpas, F. J., Romero-Puertas, M. C. McCarthy, I., & Del Rio,

L.A. (2002). Plant proteases, protein degradation, and oxidative stress: Role of Peroxisomes. *Plant Physiology and Biochemistry*. 40:521-530.

Pozo-Insfran, D., Urias-Lugo, D., Hernandez-Brenes, C., & Saldivar, S. O. S. (2012). Effect of amyloglucosidase on wort composition and fermentable carbohydrate depletion in sorghum lager beers. *Journal of the Institute of Brewing*, *110*(2), 124–132.

Pradelles, R., Vichi, S., Alexandre, H., & Chassagne, D. (2009). Influence of the drying processes of yeasts on their volatile phenol sorption capacity in model wine. *International Journal of Food Microbiology*, *135*(2), 152–157.

Prakash, O., Nimonkar, Y., & Shouche, Y. S. (2013). Practice and prospects of microbial preservation. *FEMS Microbiology Letters*, *339*(1), 1–9.

Ravimannan, N. (2016). Investigating alternative yeast storage methods. *International Journal of Advanced Research in Biological Sciences (IJARBS)*, *3*(11), 109–111.

Rosini, G., Federici, F., Vaughan, A. E., & Martini, A. (1982). Systematics of the species of the yeast genus Saccharomyces associated with the fermentation industry. *European Journal of Applied Microbiology and Biotechnology*, *15*(3), 188–193.

Russell, I., & Stewart, G. G. (1981). Liquid nitrogen storage of yeast cultures compared to more traditional storage methods. *Journal of the American Society of Brewing Chemists*, *39*(1), 19–24.

Schlander, M., Distler, U., Tenzer, S., Thines, E., & Claus, H. (2017). Purification and properties of yeast proteases secreted by *Wickerhamomyces anomalus* 227 and *Metschnikovia pulcherrima* 446 during growth in a white grape juice. *Fermentation*, *3*(1).

Sigler, M. and K. (2011). Impact of the long-term maintenance method of brewer's yeast on fermentation course , yeast vitality and beer characteristics. *Journal. Inst. Brew*, *117*(3), 383–388.

Smith, J. E., McKay, D., & Molina, R. (1994). Survival of mycorrhizal fungal isolates stored in sterile water at two temperatures and retrieved on solid and liquid nutrient media. *Canadian Journal of Microbiology*, *40*(9), 736–742.

Stingheriu, R. R. (2005). Preservation method of yeast and performance in beer fermentation. *Agroalimentary Processes and Technologies*, *11*(2), 337–344.

Suga, M., Isobe, M., & Hatakeyama, T. (2000). Cryopreservation of competent intact yeast cells for efficient electroporation. *Yeast*, *16*, 889–896.

Verbelen, P. J., Van Mulders, S., Saison, D., Van Laere, S., Delvaux, F., & Delvaux, F. R. (2008). Characteristics of high cell density fermentations with different lager yeast strains. *Journal of the Institute of Brewing*, *114*(2), 127–133.

Watanabe, D., Sekiguchi, H., Sugimoto, Y., Nagasawa, A., & Kida, N. (2018). Importance of proteasome gene expression during model dough fermentation after preservation of baker's yeast cells by freezing. *Applied and Environmental Microbiology*, *84*(12), 1–11.

Yonkova, G., Georgieva, N., Ginova, T., & Terzi, A. (2007). Biochemical processes by mashing and characterization of the fermentation of feed barley during brewing. *Specific Study & Research*, *8*(2), 179–184.

Yu, C., Reddy, A. P., Simmons, C. W., Simmons, B. A., Singer, S. W., & VanderGheynst, J. S. (2015). Preservation of microbial communities enriched on lignocellulose under thermophilic and high-solid conditions. *Biotechnology for Biofuels*, *8*(1), 206.

Zyl, W. H. Van, Bloom, M., & Viktor, M. J. (2012). Engineering yeasts for raw starch conversion. *Appl Microbiol Biotechnol*, *95*, 1377–1388.