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Research Article

Characterization of wild indigenous yeasts that can withstand different stresses from traditional fermented beverages in the Amhara region, Ethiopia

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Abstract
Yeasts that can withstand stress can generate ethanol in challenging environmental circumstances. The objective of this finding was to screen, identify, and characterize wild native yeast isolates that can withstand stress from traditional fermented beverages in the Amhara Region. Twenty-eight yeast like isolates were selected from the total 91 yeast like colonies based on morphological and biochemical assays. According to the sequence of internal transcribed spacer ITS-5.8S rRNA region, all the indigenous yeast isolates were affiliated to *Saccharomyces cerevisiae*. Tolerance to 28% ethanol was discovered four isolates (A12, A21, TJ1, and TJ6). Six isolates (A1, A15, A21, TJ3, TJ6, and T5) were found to be thermotolerant at 50°C, and sixteen isolates grew at a low pH (≥ 2.5). At 85% high sugar concentration, the isolates TJ1, TJ6, and T14 were identified as having osmotolerance. The majority of the isolates were ethanol-tolerant, thermotolerant, acid-tolerant, and osmotolerant. They were capable of fermenting glucose, maltose, sucrose, fructose, and galactose. Indigenous yeast isolates that are high-stress-tolerant and potentially beneficial were isolated in this study. This investigation demonstrated that local fermentation methods and their byproducts can serve as potential sources of yeast isolates for industrial applications.

Keywords
Ethanol-tolerance, osmo-tolerance, *Saccharomyces cerevisiae*, thermo-tolerance, wild indigenous yeasts

Abbreviations
DNA – deoxyribonucleic acid; ITS – internally transcribed spacer regions; NaCl – sodium chloride; OD – optical density; PCR – polymerase chain reaction; RPM – revolution per minute; YEPDA – yeast extract peptone dextrose agar.

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Introduction

Mankind has used the yeasts fermentative activity to produce alcoholic drinks and leavened bread for thousands of years (Noor et al. 2002). Yeasts are used widely nowadays, primarily in the chemical and food industries, healthcare, biological, biomedical, and environmental research (Camarasa et al. 2011). *Saccharomyces cerevisiae* is the yeast most commonly used for ethanol production on an industrial scale, as it tolerates a wide range of stress (Lin et al. 2014). During alcoholic fermentation, yeast is subjected to several stressful circumstances in industrial fermentation processes, including temperature, ethanol concentration, osmotic pressure, and ionic stress that affect yeast's performance and kinetics (Abdel-Banat et al. 2010; Fleet 2008; Techaparin et al. 2017).

Efficiently conversion of carbohydrates to ethanol depend on *S. cervisiae* with capable of withstanding stressful conditions and function under industrial circumstances, which is one aspect of cost-effective ethanol production (Snowdon et al., 2009). One of the more studied factors that causes stress in microorganisms is temperature changes (Abdel-Banatetal 2010; Ansanay-Galeote et al. 2001). Exothermic processes that take place during fermentation release heat, which causes the fermenter temperature to rise if the environmental temperature is already high (Attfield et al. 1992). During the fermentation of sugar, yeasts frequently face challenges related to temperature rise (35-40℃) and high concentrations of ethanol (above 20%) (Tofighi et al. 2014).

Ethiopia is one of the countries where a variety of fermented beverages made locally and consumed for a long time. These include tella, tej, keribo, korefe, buqri, shameta, borde, imbushbush, winetej, duka, and distilled spirits such as katikala or areke (Ashenafi 2008; Getnet and Berhanu 2017; Tafere 2015). Among them local Tella, Tej, and Areke are most popular traditional fermented alcoholic drinks in Ethiopia (Andualem et al. 2017; Tafere 2015). Tradition fermentation occurs naturally under the action of microorganisms associated with the raw material (Bahiru et al. 2006; Lefyedi et al. 2005; Tamminen et al. 2004). One of the promising sources of native yeasts that are resistant to stress for the production of industrial ethanol is the traditional fermented beverages.

Several studies were undertaken on the isolation of yeasts from Ethiopian traditional fermented beverages for ethanol production potential (Teramoto et al. 2005; Andualem et al. 2017; Tesfaw et al. 2021). Most of these investigations identified yeast isolates with combined tolerance to different stress conditions *in vivo*. According to Bahiru et al. (2001), traditional fermented beverages is a promising source of stress-tolerant indigenous yeasts. However, there is still a dearth of information on the characterization of stress-tolerance yeast strains from local beverages for better ethanol production than the current yield. Identifying stress-tolerant yeast isolates may help in small-scale fermentation to obtain high performance in ethanol production.

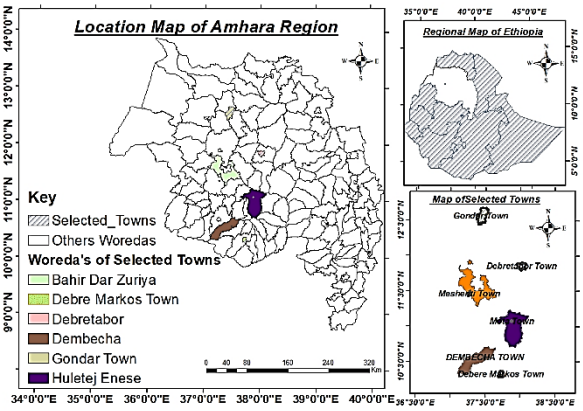

Figure 1. Map of the study area

Materials and Methods

Sample collection. Samples of the local homemade Tella, Tej, and Areke were gathered from various locations in the Amhara Region (Gondar town, Mota, Meshenti, Debre Markos, Debre Tabor, Dembecha, and surrounding villages) from October through January 2021 and 2022 (Fig. 1). Based on their high production reports, the sample locations were selected. From the total of 55 samples (Tella (n = 20), Tej (n = 15), and Areke (n = 20)) were randomly selected to be collected from five vending houses in each town and village. Samples were taken from each site and placed in sterile, screw-capped bottles (500 ml). All samples were labeled, transported in an icebox (at 4℃) to the University of Gondar at microbiology laboratory, Ethiopia, and stored in the refrigerator at 4℃ for further investigations.

Isolation of indigenous wild yeasts. Yeast was isolated from each of the Tella, Tej, and Areke samples by serial dilution plate technique on a yeast extract peptone dextrose agar (YEPDA) medium containing (yeast-extract (10), dextrose (glucose) (20), peptone (20), and agar (20), all $g.l^{-1}$). The medium was added with 50 µg chloramphenicol to inhibit bacterial growth as described by Teramoto et al. (2005). The plates were allowed to incubate for 24 to 48 h at 30℃. Morphologically distinguished colonies were then selected by dissection microscopy (Optika ST-30-2LF, Italy). The selected colonies were further subcultured to ensure their purity, and for further testing, the pure colonies were kept in glycerol 20% (v/v) at -20℃ and on YEPD agar slants at 4℃. The isolates codes from Areke were designated as A1, A3, A7, A9, A11, A12, A14, A15, A17, A18, and A21. TJ1, TJ2, TJ3, TJ4, TJ6, TJ9, and TJ0 from Tej, T1, T5, T6, T10, T11, T12, T14, T15, T17, and T18 from Tella samples for every entity to confirm the source of the organism.

Characterization of isolated yeasts

Morphological and cultural characterization. The yeast isolates were checked for their morphological characteristics after being grown on YEPD solid and liquid medium (Aila et al. 2020). Each isolate from the young culture was taken in a loopful, added to each media, and cultured for three days at 30℃. The colony morphology, such as colony color, shape, and white and creamy texture, was recorded. According to Kurtzman et al. (2011), the development of turbid, flocculent, or mucoid sediment, a ring, floating islets, or a yeast pellicle was also investigated in liquid media. The young, actively grown culture of each isolate was stained with lactophenol-cotton blue and investigated microscopically to determine ascospore presence, budding pattern, and microscopy shape (Cartwright 2010). Acetate agar (yeast extract (2.5%), dextrose (1%), potassium acetate (10%), and agar (3%)) was used to study ascospore development. After each isolate was inoculated into a sterile acetate agar medium, the samples were grown for six days at 30℃. After a 6-day period, under 40 X and 100 X objectives the cells were examined to look for the formation of ascospores (Kurtzman et al. 2005).

Sugar fermentation test. A phenol-red broth medium was employed for the sugar fermentation test, containing ($g.l^{-1}$): 1 g of beef extract, 5 g of sodium chloride, 0.081g of phenol-red, and was diluted with 1000 ml of distilled water. Then, 10% of the desired carbohydrates (glucose, sucrose, lactose, galactose, fructose, maltose, trehalose, and xylose) were added to each flask. A 10 ml container of phenol red carbohydrate broth was filled, and gas generation was detected by inserting a sterile Durham tube (30×6mm, TES1102). Then, the medium was made sterilized. After sterilization, every active yeast isolate was added to a loop, which was subsequently cultured for 24-72 h at 30℃. The medium of liquid phenol red broth changed to yellow, recognized a positive result as described by Kurtzman et al. (2011).

Physiological characterization

Detection for ethanol tolerance. Tolerance to high ethanol concentrations in YEPD broth was assessed for the isolates using 5%, 10%, 15%, 20%, 22%, 24%, 26%, and 28% of absolute ethanol, based on the procedure of (Osho 2005). Ethanol was added after sterilization 10 ml of YEPD liquid media and one ml of absolute ethanol at different concentrations (ranging from 5 to 28% v/v) was taken and transferred to YEPD liquid media. Each test tube was inoculated with a loopful of yeast isolate separately and incubated at 30℃. First optical density was measured with a Abron spectrophotometer (Spectrum Lab. 725s, India) at 600 nm and incubated for 24 to 48 h at 30℃, with uninoculated broth serving as a control. Following incubation, tubes were examined for optical density measurement against a direct proportional to cell growth (one OD of 660 nm = 1.85×10^{7}cells.ml^{-1}), and viability was also tested on YEPD Agar plates using the streak plate technique, and the results were recorded (Chansom et al. 2016).

Detection of sugar tolerance. The assessments of the sugar tolerance of yeast isolates were carried out in YEPD broth containing 40%, 50%, 60%, 70%, 80%, and 85% of different sugars (Fakruddin 2013). A loopful of yeast isolate containing 10 ml of sterilized YEPD liquid medium with appropriate amounts of various sugars was added to a test tube. The initial optical density was measured using a spectrophotometer Abron (Spectrum Lab. 725s, India) at 600 nm, and the sample was then incubated for 24 to 48 h at 30℃. As a comparison, a broth tube without inoculum was employed. After incubation,

tubes were inspected for optical density measurement evidence of growth, and viability was also checked on YEPD Agar plates.

Detection of thermo-tolerance. The temperature tolerance of the yeast isolates was determined on YEPD medium. A loop full of each isolate was inoculated in 10 ml of YEPD broth. A spectrophotometer Abron (Spectrum Lab. 725s, India) was used to measure the initial optical density of each test tube at 600 nm. The tubes were then incubated for 24 to 48 h at various temperatures (35, 40, 45, 50, and 55℃). As a comparison, a broth tube without inoculum was used. After incubation, tubes were examined for proof of their growth by using optical density measurement, and viability was also observed on YEPD Agar plates to make sure features of thermotolerance (Priya et al. 2016).

Detection of salt-tolerance. Salt tolerance was measured in YEPD broth with NaCl concentrations of 10%, 15%, 20%, 25%, 30%, and 35% (w/v). Each isolate was inoculated at the required concentration of NaCl. The initial optical density was measured using a spectrophotometer spectrophotometer Abron (Spectrum Lab. 725s, India) at 600 nm, and incubated at 30℃ for 24 to 48 h, with uninoculated broth serving as a control. After incubation, tubes were looked for growth by using optical density measurement, and viability was also observed on YEPD Agar plates.

Detection of pH-tolerance. The pH tolerance of the yeast isolates was evaluated using a YEPD broth medium that adjusted to different pH values (2.5, 3.0, 3.5, and 4.0). Each isolate was inoculated a loopful into test tubes containing 10 ml of sterilized YEPD liquid media. Each test tube initial optical density was measured at 600 nm using an spectrophotometer Abron (Spectrum Lab. 725s, India), and the tubes were then incubated for 24 to 48 h at 30℃. Uninoculated medium was also used as a control. After incubation, tubes were examined for optical density measurement for evidence of growth, and viability was also checked on YEPD Agar plates (Priya et al. 2016).

Genomic DNA extraction and PCR Amplification. Genomic DNA was isolated from each yeast isolate and used as a template for PCR. Pure yeast isolates were grown for 2 days at 30℃ in a YEPD medium. The pellets of yeast were collected at 6.037 g using centrifugation (model 280-R, China). In accordance with the instructions of the manufacture, genomic DNA was extracted using the Gene EluteTM Plant Genomic DNA Purification Kit (Sigma-Aldrich, Burlington, MA). The pelleted cells were kept in a deep freezer at -20℃ until they were required. To confirm the DNA's purity, the genomic DNA was examined using a nanodrop and gel electrophoresis. The 5.8S rRNA gene and the universal primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) (Sigma Company) were amplified using method (White et al. 1990). A 40 µl reaction volume was used for the PCR containing DNA template (4 µl), each primer (1µl) from TSINGKE Biological Technology Co., Ltd. (Beijing, China) at a final concentration of 0.25 $\mu M.\mu l^{-1}$, 5X FIREPol Master Mix (8 µl) (European Solis BioDyne, Riia, Tartu, Estonia), and deionized water (26 µl). Gelelectrophoresis of the PCR products was performed on 1% agarose gel using 1XTris-acetate-EDTA (TAE) buffer obtained from Blulux Laboratories Pvt. Ltd. Faridabad, India. After staining the gels with ethidium bromide, samples were examined using a UV detector. The molecular weight marker 100 bp DNA ladder (HiMedia Laboratories Pvt. Ltd, SwastikDisha Business Park, Mumbai, India) was also used to calculate the molecular sizes of the amplicons. For sequencing, the PCR products sent to the Macrogen Europe BV Laboratory in Amsterdam, The Netherlands.

Sequence analysis and phylogenetic tree construction. Sequenced data were modified with the BioEdit software, and their similarity to previously deposited sequences from GenBank databases was assessed using the BLAST search engine. Using the National Center for Biotechnology Information's taxonomy browser, the isolates' sequences were further matched and compared to ITS sequences in the database (NCBI). ClustalW was used to align 44 sequences (22 from the database and 22 from this study) to construct phylogenetic trees (Larkin et al. 2007). The maximum likelihood (ML) algorithm character estimation approach with bootstrap assessments with 1000 repetitions was used for the molecular evolutionary genetics analysis (MEGA11) (Kumar et al. 2016).

Data analysis. Characterization of indigenous wild yeasts was conducted in triplicate. Phylogenetic tree construction was carried out using MEGA11 software.

Results and Discussion

Isolation of yeasts. A total of 55 traditional fermented beverage samples (Tella, Tej, and Areke) were obtained from various locations in the Amhara Region, Ethiopia. From the total of 91 colonies, 28 morphologically distinct colonies were recovered that showed their yeast-like colonies. The majority of the yeast (11, or 39.3%) were isolated from Areke, while only 7 (25% of the total) were isolated from Tej using YEPD (Table 1). Out of the total, 28 yeast isolates were chosen after screening as representative of local Tella, Tej, and Areke samples based on their morphology and sugar fermentation test for further physiological characterization evaluations. Eleven isolates from Tella, seven isolates from Tej, and ten isolates from Areke as shown in Table 1.

The morphological features of the yeast isolates. The isolates of wild yeast were showed variations in morphological characteristics in their colony shape, texture, elevation, color, and cell shape (Table 1). Based on the morphological characteristics of colonies on YEPD solid media, majority of isolates of yeast were creamy white, smooth, small, and flat, and some had rough, raised, butyrous colony morphology. From a total of 28 isolates, 26 (92.9%) were smooth, and the rest 2 (7.1%) were exhibited rough colonies. The cell morphology of yeast isolates showed that 18 (64.3%) of colonies were spherical, 5 (17.9%) were ovoid, and 4 (14.3%) were elongated. The two isolates, A1 and TJ2 were a rough texture with flocculent formation on liquid media. Moreover, microscopic analysis revealed that all yeast isolates that reproduced via budding and ascospores were produced single, double, or triple budding cells but they were not developed pseudohyphae at all.

All of the yeast isolates exhibited the same growth pattern after three days of incubation at 30℃ in a liquid medium. On the YEPD broth medium surface, 16 (57.1%), 3 (10.7%) and 9 (32.1%) of isolates were shown precipitates, uniform turbids, and flocculents, respectively.

All isolates except A1, A9, A14, A17, TJ2, TJ3, TJ4, TJ9, TJ0, T1, T11, and T12 were developed precipitate. A1, A9, A14, TJ2, TJ4, TJ9, TJ0, T1, and T12 isolates have been exhibited flocculent properties (Table 1). All yeast isolates were not shown any pellicle formation, but uniform turbidity was observed by A17, TJ3, and T11 isolates.

Sugar utilization. The sugar utilization capability of yeast isolates was determined based on their gas production in Durham test tubes after 24h of fermentation (Table 2). Most of yeast isolates were able to utilize many of the carbon substrates; however, their degree of assimilation was differed. All of the isolates were unable to grow on xylose, trehalose, or lactose (Table 2). Isolates such as A11, A17, A18, A21, TJ1, TJ6, T6, T17, and T18 were the most adaptable, growing on more than 60% of the carbon substrates.

The isolates were showed high to moderate CO_2 gas production capacity in glucose and fructose except for A3, TJ2, TJ9, and TJ0. In sucrose, all yeast isolates were produced moderate-to-low levels of CO2 gas; while isolates A7, A17, TJ1, T5, T6, T15, and T17 were released high levels of CO2 gas in the Durham tube. Except A11, A17, A18, A21, TJ1, TJ6, T6, T17, and T18 isolates, the rest were not metabolized galactose at all. When compared to other carbon sources, all of the yeast isolates were moderate maltose and sucrose fermentation capabilities. The yeast isolates A1, A7, A11, A15, A17, A18, A21, TJ1, TJ6, T5, T6, T15, and T17 were produced high levels of CO2 gas and fermented many of the provided carbohydrates (Table 2).

Ethanol, sugar, and temperature tolerance. The ethanol tolerance of 28 yeast isolates obtained from Tella, Areke, and Tej at 15%, 20%, 22%, 24%, 26%, and 28% (v/v) ethanol concentrations were presented on (Table 3). Among 28 yeast isolates, 24, 14, and 4 of them shown tolerance to 24%, 26%, and 28% of ethanol, respectively. Increasing the absolute concentration of ethanol was inhibited the growth of yeast isolates. Almost all isolates of yeast were showed intensive growth at 15% and 20% of ethanol, while growth was moderate to low at 22% and 24% of ethanol, except in the A9, A15, TJ9, and TJ0 isolates. In the YEPD medium, isolates A12, A14, A17, A18, A21, TJ1, TJ2, TJ3, TJ4, TJ6, T12, T15, and T18 were able to grow at 26% ethanol. The

Table 1. The morphological features of the yeast isolates from Tella, Tej and Areke sample in different places of Amhara region

Yeast isolate Code	Morphology in solid media	Shape	Vegetative growth	Pseudo hyphae	Ascospore	Growth in broth
A1	Rough, Flat, Small, Creamy	Elongate	Budding	-ve	Multi polar	Flocculent
A3	Smooth, Small, Raised, Butyrous	Ovoid	Budding	-ve	Single	High Precipitate
A7	Smooth, Raised, White creamy	Ovoid	Budding	-ve	Single	High Precipitate
A9	Smooth, Flat, Small, Creamy	Spherical	Budding	-ve	Multi polar	Flocculent
A11	Smooth, Large, Raised, Creamy	Elongate	Budding	-ve	Bi polar	High precipitate
A12	Smooth, Raised, Small, White creamy	Spherical	Budding	-ve	Single	High Precipitate
A14	Smooth, Flat, Small, Creamy	Spherical	Budding	-ve	Single	Flocculent
A15	Smooth, Raised, Small, White creamy	Spherical	Budding	-ve	Multi polar	High Precipitate
A17	Smooth, Flat, Small, Creamy	Spherical	Budding	-ve	Bi polar	Uniform Turbid
A18	Smooth, Raised, Small, White creamy	Spherical	Budding	-ve	Multi polar	High Precipitate
A21	Smooth, Flat, White creamy	Spherical	Budding	-ve	Single	High Precipitate
TJ1	Smooth, Flat, Small, Butyrous	Spherical	Budding	-ve	Multi polar	High Precipitate
TJ2	Rough, Flat, Small, Creamy	Elongate	Budding	-ve	Single	Flocculent
TJ3	Smooth, Flat, Small, Creamy	Elongate	Budding	-ve	Bi polar	Uniform Turbid
TJ4	Smooth, Flat, Small, Butyrous	Spherical	Budding	-ve	Multi polar	Flocculent
TJ6	Smooth, Flat, Small, White creamy	Spherical	Budding	-ve	Bi polar	High Precipitate
TJ9	Smooth, Flat, Large, Creamy	Spherical	Budding	-ve	Single	Flocculent
TJ0	Smooth, Flat, Large, White creamy	Spherical	Budding	-ve	Multi polar	Flocculent
T1	Smooth, Raised, Large, White Creamy	Spherical	Budding	-ve	Multi polar	Flocculent
T5	Smooth, Flat, Small, Butyrous	Ovoid	Budding	-ve	Single	High Precipitate
T6	Smooth, Raised, Small, Creamy	Spherical	Budding	-ve	Single	High Precipitate
T10	Smooth, Flat, Large, Creamy	Spherical	Budding	-ve	Single	Low Precipitate
T11	Smooth, Flat, Large, White creamy	Ovoid	Budding	-ve	Multi polar	Uniform Turbid
T12	Smooth, Flat, Small, Creamy	Spherical	Budding	-ve	Multi polar	Flocculent
T14	Smooth, Raised, Small, Creamy	Spherical	Budding	-ve	Multi polar	High Precipitate
T15	Smooth, Flat, Small, Creamy	Spherical	Budding	-ve	Single	Low Precipitate
T17	Smooth, Flat, Small, Creamy	Ovoid	Budding	-ve	Single	High Precipitate
T18	Smooth, Flat, Large, Butyrous	Spherical	Budding	-ve	Single	Low Precipitate

least ethanol tolerant were A3, A9, A15, TJ9, and T15 to an ethanol concentration of 22%, followed by A1, A3, A7, A11, A17, TJ1, T1, T5, T10, T11, T14 and T17, which were able to grow to 24% ethanol in the medium. The only isolates which able to grow in 28% ethanol concentration were isolates of A12, A21, TJ1, and TJ6. Above 28% ethanol concentration, none of the isolates of yeast were found to be tolerant.

Yeast isolates growth at various concentrations of glucose (50%, 60%, 70%, 80%, and 85%) is shown in (Table 3). All yeast isolates were showed disparities in tolerance to varying sugar amounts. Sugar-tolerant yeast isolates growth were increased at concentration up to 50% but gradually decreased up to concentration of 85%. All the isolates were grown on media containing 50–60% glucose and among them TJ1, TJ6, and T14 isolates were able to grow up to 85% sugar concentration.

The temperature-tolerant yeast isolates' growth results at 40℃, 45℃, 50℃, and 55℃ are presented in Table 3. Most isolates were indicated significant ($P \leq 0.05$) variations in their capacity to grow in a medium with a temperature of 40℃ to 50℃ on the YEPD medium. At 40℃, all yeast isolates were grown. Among the isolates, only A1, A15, A21, TJ3, TJ6, and T5 were able to grow the temperature of at 50℃. At 45℃, moderate growth was observed in yeast isolates (A1, A15, A21, TJ3, and TJ6) compared to the other isolates.

Table 2. Sugar utilization profile of yeast isolates at 30℃ YEPD medium using Durham tube

Carbon Source	**Isolates**													
Sugar profile	**A1**	**A3**	**A7**	**A9**	**A11**	**A12**	**A14**	**A15**	**A17**	**A18**	**A21**	**TJ1**	**TJ2**	**TJ3**
Glucose	+++	+	+++	++	+++	+++	++	+++	+++	+++	+++	+++	+	++
Galactose	-	-	-	-	+	-	-	-	+	+	+	+	-	-
Maltose	++	+	++	++	++	++	++	++	++	++	++	++	+	++
Sucrose	++	+	+++	++	++	++	++	++	+++	++	++	+++	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+++	++	++	+++	+++	++	++	+++	++	++	+++	+++	+	++
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TJ4	**TJ6**	**TJ9**	**TJ0**	**T1**	**T5**	**T6**	**T10**	**T11**	**T12**	**T14**	**T15**	**T17**	**T18**
Glucose	++	+++	+	++	++	+++	+++	++	++	++	+++	+++	+++	+++
Galactose	-	+	-	-	-	-	+	-	-	-	-	-	+	+
Maltose	++	++	+	+	+	++	++	++	++	+	++	+	++	++
Sucrose	++	++	+	++	++	+++	+++	++	+	++	++	+++	+++	++
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+++	+++	+	+	++	++	+++	++	++	++	++	++	+++	++
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+++) high CO_2 gas production, (++) medium CO_2 gas production, (+) low CO_2 gas production and (-) no CO_2 gas production

Salt, and pH tolerance. The ability of yeast isolates to tolerate different NaCl concentrations in YEPD media was demonstrated in Table 4. Yeast isolates were grown at different salt concentrations (20%, 25%, 30%, and 35%), and the growth of the isolates varied at each concentration. All isolates were grown in YEPD medium with salt concentrations of 20% and 25% NaCl. Yeast isolates such as A12, A14, A17, A18, A21, TJ2, TJ6, T5, T10, T12, T17, and T18 were shown to have moderate growth compared to all other isolates at 25% NaCl. Yeast isolates A12, A14, A17, A18, A21, TJ2, TJ3, TJ4, TJ6, T5, T6, T12, T14, T17, and T18 were shown to be tolerant up to 30% NaCl in comparison with others. With a 35% NaCl concentration, no isolate was able to grow. Indeed, at a 30% NaCl concentration, the percentage of survival was 15 (53.5%).

Table 3. Growth of yeast isolates from Tella, Tej and Areke under various ethanol, sugar concentrations and temperatures

Ethanol conc., % (v/v)	**A1**	**A3**	**A7**	**A9**	**A11**	**A12**	**A14**	**A15**	**A17**	**A18**	**A21**	**TJ1**	**TJ2**	**TJ3**
15	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
20	+++	++	+++	++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
22	++	+	++	+	++	++	++	+	++	++	+++	++	++	++
24	+	+	+	_	+	++	+	_	+	++	++	++	+	++
26	_	_	_	_	_	+	+	_	+	+	+	+	+	+
28	_	_	_	_	_	+	_	_	_	_	+	+	_	_
	TJ4	**TJ6**	**TJ9**	**TJ0**	**T1**	**T5**	**T6**	**T10**	**T11**	**T12**	**T14**	**T15**	**T17**	**T18**
15	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
20	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
22	+++	+++	+	+	++	++	+++	++	++	++	++	++	++	++
24	++	++	_	_	+	+	++	+	+	++	+	+	+	+
26	+	+	_	_	_	_	+	_	_	+	_	+	_	+
28	_	+	_	_	_	_	_	_	_	_	_	_	_	_
Sugar conc., % (v/v)	**A1**	**A3**	**A7**	**A9**	**A11**	**A12**	**A14**	**A15**	**A17**	**A18**	**A21**	**TJ1**	**TJ2**	**TJ3**
50	+++	++	+++	++	+++	+++	+++	+++	++	+++	++	+++	+++	+++
60	+++	+	++	+	++	++	++	++	++	++	+	++	++	++
70	++	_	+	_	+	++	+	+	_	+	_	++	+	+
80	+	_	_	_	_	+	_	_	_	_	_	+	_	_
85	_	_	_	_	_	_	_	_	_	_	_	+	_	_
	TJ4	**TJ6**	**TJ9**	**TJ0**	**T1**	**T5**	**T6**	**T10**	**T11**	**T12**	**T14**	**T15**	**T17**	**T18**
50	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
60	++	++	+	+	++	++	+++	++	++	++	++	++	+++	++
70	+	++	_	+	+	+	++	+	+	+	++	+	++	+
80	+	+	_	_	_	+	+	_	_	+	+	_	+	_
85	_	+	_	_	_	_	_	_	_	_	+	_	_	_
Temperature														
	A1	**A3**	**A7**	**A9**	**A11**	**A12**	**A14**	**A15**	**A17**	**A18**	**A21**	**TJ1**	**TJ2**	**TJ3**
40°C	++	++	++	++	++	++	++	+++	++	++	++	++	++	+++
45°C	++	_	+	+	+	++	+	++	+	+	++	+	+	++
50°C	+	_	_	_	_	_	_	+	_	_	+	_	_	+
55°C	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	TJ4	**TJ6**	**TJ9**	**TJ0**	**T1**	**T5**	**T6**	**T10**	**T11**	**T12**	**T14**	**T15**	**T17**	**T18**
40°C	++	+++	+++	++	++	++	++	++	++	++	++	++	++	++
45°C	+	++	+	+	+	+	+	+	_	+	+	+	+	+
50°C	_	+	_	_	_	+	_	_	_	_	_	_	_	_
55°C	_	_	_	_	_	_	_	_	_	_	_	_	_	_

+++ (Intensive growth), ++ (Moderate growth), + (Little growth), - (No growth)

The capacity of yeast isolates to tolerate various pH scales on YEPD media was described in Table 4. Different pH values (2.5, 3.0, 3.5, and 4.0) were cultured yeast isolates, and the growth of the isolates were showed significant ($p \leq 0.05$) variations at these values. The yeast isolate's growth were increased from 2.5 to 4.0 pH. Isolates such as A1, A3, A9, A15, A17, A18, TJ6, TJ0, T5, and T15 were showed intensive to moderate growth at 3.5 and 4.0 pH.

Table 4. Growth of yeast isolates from Tella, Tej and Areke under various salt and pH values

Salt tolerance	**A1**	**A3**	**A7**	**A9**	**A11**	**A12**	**A14**	**A15**	**A17**	**A18**	**A21**	**TJ1**	**TJ2**	**TJ3**
20	++	+	++	++	++	++	+++	++	+++	+++	+++	++	++	+++
25	+	+	+	+	+	++	++	+	++	++	++	+	++	+
30	_	_	_	_	_	+	+	_	+	+	+	_	+	+
35	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	TJ4	**TJ6**	**TJ9**	**TJ0**	**T1**	**T5**	**T6**	**T10**	**T11**	**T12**	**T14**	**T15**	**T17**	**T18**
20	++	+++	+	+	++	++	++	++	+	+++	++	++	++	+++
25	+	++	+	+	+	++	+	++	+	++	+	+	++	++
30	+	+	_	_	_	+	+	_	_	+	+	_	+	+
35	_	_	_	_	_	_	_	_	_	_	_	_	_	_
pH tolerance	**A1**	**A3**	**A7**	**A9**	**A11**	**A12**	**A14**	**A15**	**A17**	**A18**	**A21**	**TJ1**	**TJ2**	**TJ3**
2.5	_	_	+	_	_	+	+	_	_	+	_	+	+	+
3.0	+	+	++	+	+	+	++	+	+	+	+	+	++	+
3.5	+	+	++	++	++	++	++	+	+	+	++	++	+++	++
4.0	++	++	+++	+++	++	+++	+++	++	++	++	+++	+++	+++	+++
	TJ4	**TJ6**	**TJ9**	**TJ0**	**T1**	**T5**	**T6**	**T10**	**T11**	**T12**	**T14**	**T15**	**T17**	**T18**
2.5	+	+	_	_	+	_	+	+	+	_	+	_	+	+
3.0	++	+	+	+	+	+	++	+	+	+	+	+	+	++
3.5	+++	++	+	+	++	+	++	++	++	++	++	+	++	++
4.0	+++	++	++	++	++	++	+++	++	++	+++	+++	++	++	+++

+++ (Intensive growth), ++ (Moderate growth), + (Little growth), - (No growth)

Molecular identification of the isolates. Each yeast isolates amplified PCR product were showed a length of between 504 and 920 bp based on the given ladder (Table 6). According to this investigation, all isolates were found affiliated to be *Saccharomyces cerevisiae* (>98%) (Table 5). The sequence of 22 isolate was deposited at GeneBank with their accession numbers summarized in Table 6. According to the phylogenetic tree, *Saccharomyces cerevisiae* IFO 1833 and *Saccharomyces cerevisiae* IFO1046 shared one clade cluster with 100% similarity (Fig. 2). *Saccharomyces cerevisiae* IFO 1833, *Saccharomyces cerevisiae* IFO1046, and *Saccharomyces cerevisiae* isolate NG-LCU-P1(2) shared a single cluster of clades with 98% similarity.

The biochemical test of the yeast isolate was determined by the fermentation patterns of carbon sugars. The majority of sugars, including glucose, maltose, sucrose, fructose, galactose, and xylose, are positive in the fermentation process, as evidenced by acid production due to color change from pink to yellow.

These results were in line with the previous findings (Andualem et al. 2017; Kumar et al. 2011; Thapa et al. 2015). Each yeast isolate used in this investigation were consumed six-carbon sugar. This may serve as a crucial signal that isolates are required to transform the provided sugars into essential products like ethanol. Similar findings were reported by Kumar et al. (2011), who found the majority of isolates were able to ferment glucose, galactose, maltose, and sucrose.

Table 5. Identification of yeast isolates based on 5.8S-ITS rDNA comparative sequence

Isolates code	Sample origin	Closest relative with their accession number and percent of identity		
		Closest relative	Accession Number	Identity (%)
A1	Areke	*Saccharomyces cerevisiae* IFO 1046	**LC576586.1**	99.87%
A3	Areke	*Saccharomyces cerevisiae* isolate j3836sh	**KP204934.1**	99.61%
A11	Areke	*Saccharomyces cerevisiae* isolate M9	**KP723680.1**	99.87%
A12	Areke	*Saccharomyces cerevisiae* strain CNMN-Y-33	**MT641207.1**	99.67%
A14	Areke	*Saccharomyces cerevisiae* strain CNMN-Y-32	**MT641203.1**	98.99%
A15	Areke	*Saccharomyces cerevisiae* IFO 1833	**LC576590.1**	99.87%
A18	Areke	*Saccharomyces cerevisiae* strain CNMN-Y-31	**MT640330.1**	99.65%
TJ1	Tej	*Saccharomyces cerevisiae* isolate BARBM_16.2	**OP663245.1**	99.61%
TJ2	Tej	*Saccharomyces cerevisiae* strain T15-20	**MT522376.1**	99.82%
TJ3	Tej	*Saccharomyces cerevisiae* isolate NG-LCU-P1(2)	**OR128359.1**	99.86%
TJ6	Tej	*Saccharomyces cerevisiae* isolate OM20	**MT136553.1**	99.82%
TJ9	Tej	*Saccharomyces cerevisiae* isolate L26A	**KP723679.1**	99.48%
TJ0	Tej	*Saccharomyces cerevisiae* strain LY183	**KY711301.1**	99.23%
T5	Tella	*Saccharomyces cerevisiae* strain F6	**ON738691.1**	98.34%
T6	Tella	*Saccharomyces cerevisiae* isolate E21567	**MK267684.1**	98.29%
T10	Tella	*Saccharomyces cerevisiae* isolate B-WHX-12-43	**KC544486.1**	99.74%
T11	Tella	*Saccharomyces cerevisiae* isolate JGF28	**MZ089545.1**	99.61%
T12	Tella	*Saccharomyces cerevisiae* IFO 1833	**LC576590.1**	99.48%
T14	Tella	*Saccharomyces cerevisiae* strain CNMN-Y-33	**MT641207.1**	99.67%
T15	Tella	*Saccharomyces cerevisiae* isolate F62	**MN944498.1**	98.81%
T17	Tella	*Saccharomyces cerevisiae* isolate 10-1358	**MF375633.1**	99.87%
T18	Tella	*Saccharomyces cerevisiae* isolate BARBM_16.2	**OP663245.1**	98.44%

*Strains accession number obtained from searching the NCBI database

One of the preferred qualities of yeast isolates for production of ethanol is their capacity to tolerate high ethanol concentrations. Ethanol is the primary stress that causes decreased ethanol production and stuck fermentation by influencing yeast growth and fermentation rate (Gibson et al. 2007; Stanley et al. 2010). Each isolate in the present finding was shown to withstand more than 20% ethanol. Ethanol is commonly known that an inhibitor of the growth of microbes, however, yeast isolates A12, A21, TJ4, and TJ6 were able to tolerate ethanol concentrations at a higher level of 28% v/v compared to the other isolates. Similarly reported by Negera (2017) ethanol concentrations of 15 to 25% (v/v), the yeast isolates AAUP1, AAUD, AAUG3, and AAUAV were able to grow. According to Fakruddin et al. (2013) thermotolerant yeasts C, T, and DB2 were grown at 0-20% (v/v) ethanol concentration, whereas *Saccharomyces cerevisiae* isolated from palm wines tolerated the highest-level ethanol concentration up to 24% v/v (Nwachukwu et al. 2006). Local Tella, Tej, and Areke can be the best yeast isolate sources and may be used in the future in the manufacture of commercial ethanol and bioethanol. Additionally, the results of this investigation indicate a promising source of ethanol-tolerant noble yeast isolates.

Table 6. Isolate code, name given to 22 isolates for submission, and Genbank accession numbers

Isola-tes code	Sample origin	Code given to isolates for submission	Length of PCR product, bp	Genbank accession number
A1	Areke	*Saccharomyces cerevisiae* isolate MUA1F	783	**OR209275.1**
A3	Areke	*Saccharomyces cerevisiae* isolate MUA3F	798	**OR209274.1**
A11	Areke	*Saccharomyces cerevisiae* isolate MUA11F	796	**OR209277.1**
A12	Areke	*Saccharomyces cerevisiae* isolate R9MU	604	**OR143320.1**
A14	Areke	*Saccharomyces cerevisiae* isolate MUA14F	593	**OR209281.1**
A15	Areke	*Saccharomyces cerevisiae* isolate MUA15F	809	**OR209287.1**
A18	Areke	*Saccharomyces cerevisiae* isolate R20MU	570	**OR143322.1**
TJ1	Tej	*Saccharomyces cerevisiae* isolate R19MU	920	**OR143321.1**
TJ2	Tej	*Saccharomyces cerevisiae* isolate MUTJ6F	553	**OR186523.1**
TJ3	Tej	*Saccharomyces cerevisiae* isolate MUTJ3F	702	**OR209279.1**
TJ6	Tej	*Saccharomyces cerevisiae* isolate MUTJ6F	552	**OR186523.1**
TJ9	Tej	*Saccharomyces cerevisiae* isolate MUTJ29F	794	**OR186524.1**
TJ0	Tej	*Saccharomyces cerevisiae* isolate MUTJ0F	782	**OR209280.1**
T5	Tella	*Saccharomyces cerevisiae* isolate MUT5F	602	**OR209282.1**
T6	Tella	*Saccharomyces cerevisiae* isolate MUT6F	761	**OR209283.1**
T10	Tella	*Saccharomyces cerevisiae* isolate MUT10F	500	**OR209284.1**
T11	Tella	*Saccharomyces cerevisiae* isolate R24MU	798	**OR143323.1**
T12	Tella	*Saccharomyces cerevisiae* isolate MUT12F	764	**OR209278.1**
T14	Tella	*Saccharomyces cerevisiae* isolate R21MU	591	**OR143319.1**
T15	Tella	*Saccharomyces cerevisiae* isolate MUT15F	504	**OR209276.1**
T17	Tella	*Saccharomyces cerevisiae* isolate MUT17F	755	**OR209285.1**
T18	Tella	*Saccharomyces cerevisiae* isolate MUT18F	882	**OR209286.1**

Accession number and code for strains were deposited at GeneBank

Industries typically ferment at a temperature of 25 to 35℃, but during fermentation, the temperature might rise over 40℃, which reduces the viability and productivity of the cells. The benefits of using thermotolerant yeasts during ethanol production has recovery of ethanol by considerable reductions in refrigeration control running expenditures in alcohol distilleries (Aguilera et al. 2007; Fonseca et al. 2008). In this study, six yeast isolates (A1, A15, A21, TJ3, TJ6, and T5) were found to be thermotolerant, as they were able to grow at 50ºC. Earlier investigations have shown that certain thermotolerant yeast strains can ferment and generate ethanol efficiently at temperatures between 38-45℃ and grow efficiently at temperatures as high as 45-52℃ (Ballesteros et al. 2004; Limtong et al. 2007). The findings in this study documented industrially interesting and promising yeast isolates that survive temperature-stress environment for better ethanol production.

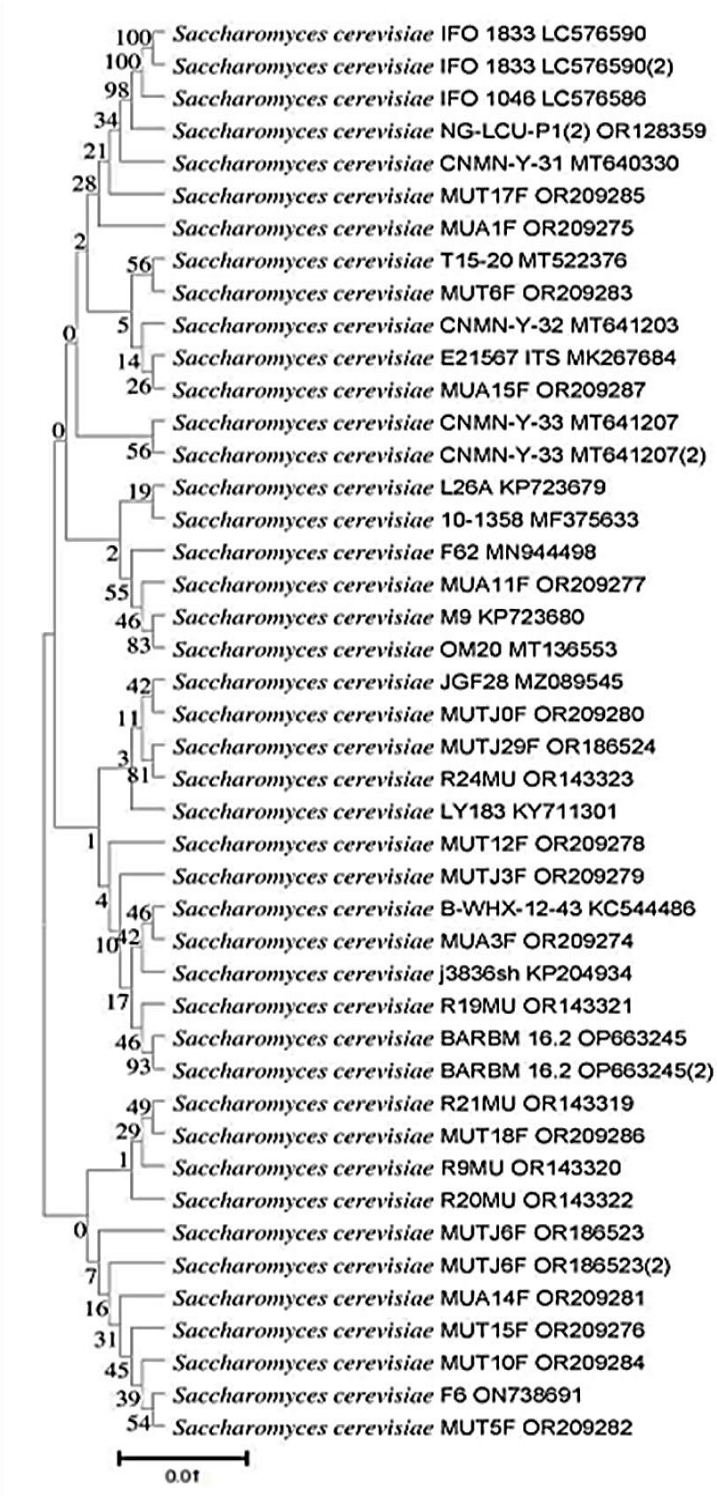

Figure 2. The ITS sequences' phylogenetic tree was created using MEGA 11 software. ClustalW was used to align 44 sequences (22 from the database and 22 from this study) to construct phylogenetic trees. To draw the phylogenetic tree, a bootstrap value of 1000 was utilized. The sequences were aligned by using the neighbour joining method and maximum likelihood estimation. Bar, 0.01% difference in nucleotide sequence

The sugar pressure in high-gravity worts results in a high osmotic potential, which may alter yeast metabolism, reduce yeast viability, and enhance the toxicity caused by ethanol (Deesuth et al. 2016). Industrial applications require yeasts that can endure stress and continue to be viable throughout the fermentation process (Tofighi et al. 2014). In the present study, all yeast isolates showed intensive to moderate growth at 50-60% sugar. According to the findings of the current investigation, yeast isolates (A1, A12, TJ1, TJ4, TJ6, T5, T6, T12, T14, and T17) were tolerant of high glucose concentrations at 80%, while isolates (TJ1, TJ6, and T14) were also shown to be resistant to high sugar concentrations at 85%. The results of this investigation show that yeast isolates with better tolerance than previously reported by Ok and Hashinaga (1997) all isolates except two were grew on media containing 50% (w/w) glucose. One strain, however, continued to grow in a liquid medium with a glucose concentration of 80% (w/w). The findings of the present study showed that the presence of strong sugar tolerance yeast isolates may be an annoying problem in beverage preservation.

Yeast isolates were exhibited growth in the range between pH 2.5 and 4.0. However, pH 2.0 or below was unable for any of them to grow. According to Periyasamy (2009), the maximum amount of ethanol produced by isolates grown in the 2.0-5.0 pH range was at pH 4.0. Similar research revealed that *Saccharomyces* grows efficiently in the pH range of 2.8 to 4.2, and below pH 2.8 fermentation and growth were inhibited (Arroyo-López et al. 2009). This is probably because of the optimum pH value for the action of proteins attached to plasma membranes, such as transport proteins and enzymes (Lopandic et al. 2006). In the current study, the presence of acidophilus yeasts may be important to reduce bacterial contamination during the fermentation of ethanol.

At 25% NaCl, All yeast isolate were showed moderate to little growth, and some of the isolates showed higher tolerance up to 30% NaCl. This result contradicted previous reports that yeast strains found in palm wine shown a salt tolerance of up to 20% NaCl. However, two strains (*S. cerevisiae* PWB13 and *Kluyueromyces africanus* PWA21) and one strain (*S. cerevisiae* PWB13) were able to withstand 15% and 20% NaCl, respectively (Lopandic et al. 2006; Walker 2009).

Internal transcribed spacer section (ITS-5.8S rRNA) sequencing is a useful technique for species identification and evolutionary studies (Schoch et al. 2012). Based on the ITS-5.8S rRNA sequencing, yeasts from domestic beverages (Tella, Tej, and Areke) were affiliated to *Saccharomyces cerevisiae* in this study. Consistent with the current findings, Beyene et al. (2020) used the ITS-5.8S rRNA region to determine that the native alcoholic beverage (Tej and shamita) contains the yeast species *Saccharomyces cerevisiae*. Likewise, Koricha et al. (2020) have identified the yeast species *Saccharomyces cerevisiae* based on ITS-5.8S rRNA region by sequence analysis from Ethiopian fermented beverages.

Conclusions

According to the findings of this study, local Tella, Tej, and Areke can be potential sources of yeast isolates that can be utilized for the production of commercial ethanol. Moreover, the results of this investigation provide a potential source of wild yeast isolates that are noble and high-stresstoleranc e. Some of the isolates had grown in a wider range of ethanol, temperature, sugar, salinity, and carbon sources. They also tolerate low pH levels, which is also a desired feature. A wild-type of yeast isolates A12, A15, A21, TJ1, TJ3, TJ6, T5, T6, and T14 obtained in this study can be used as a potential candidate for beer and bioethanol industri es.

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