Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Bioethanol production using stress-tolerant yeast strains isolated from (Areke) and sugar beet (Beta vulgaris) juice (as substrate)

Mulugeta Fentahun

mulufenta@gmail.com

Debre Markos University

Article

Keywords: Areke, Bioethanol, Optimization, Sugar beet juice, Yeast strains

Posted Date: July 24th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7192351/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on April 2nd, 2026. See the published version at https://doi.org/10.1038/s41598-026-45172-2.

Abstract

Traditional fermented beverages can be a potential source for industrial microorganisms. Among alternative energy sources, biofuel has been gaining increasing attention as a replacement for fossil fuels. This study aimed to investigate improved bioethanol production under optimal conditions using yeast strains isolated from a local beverage (*areke*) and sugar beet juice as substrates. In the current study, four wild yeast strains capable of withstanding multiple stresses were isolated from *areke*. Fresh sugar beets were gathered from a local supplier in Debre Markos town, and the physicochemical characterization of the sugar beet juice was determined using standard methods. Ethanol production was optimized using four selected *Saccharomyces cerevisiae* strains, namely, MUA1F (OR209275.1), MUA3F (OR209274.1), MUA11F (OR209277.1), and MUA15F (OR209276.1) using sugar beet juice as substrates. Initial pH 4.5, 20 °Brix, 30°C, 4.0 g/l NH_4SO_4, yeast extract as a supplement, and 72 hours of incubation were optimal for enhancing ethanol production. Ammonium sulfate at 4.0 g/l, 12.49 ± 0.22% v/v ethanol was produced under these optimal circumstances. These findings suggest that *Saccharomyces cerevisiae* isolates MUA3F and MUA15F should be further assessed, improved, and optimized for industrial bioethanol production due to their potential for fermentation.

Introduction

Biofuel produced from biomass is the primary fuel that replaces petroleum[1]. Renewable biomass fuels reduce emissions of greenhouse gases, efficiency of energy, and biodegradable than fossil fuels[2,3]. Moreover, due to the expensive price of fossil fuels, biofuel production has been gaining increasing attention[4]. Sugar beet juice is gaining more attention in the area of bioethanol production due to its clean and highly fermentable sugar content with minimal impurities or fermentation inhibitors that are suitable for direct fermentation[5,6]. This makes it an ideal substrate for getting high ethanol yields without requiring a lot of pretreatments compared to molasses. Research on sugar beet juice has the potential to be a valuable feedstock in integrated biorefineries for the sustainable production of biofuels, biochemicals, and other value-added products[7,8].

Indigenous fermented drinks are considered to be a significant source of yeasts that produce ethanol, which can withstand a variety of stressful situations, such as lack of nutrients and complexity, low pH, temperature fluctuations, and high osmotic stress[9–13]. Several reports state that indigenous fermented drinks are an important habit for yeasts in general and ethanol producing yeasts in particular[14]. One of the challenges of bioethanol production is obtaining efficient microorganisms that can tolerate a wide range of stress conditions. This urged searching for robust ethanologenic yeasts that can meet the demands of the bioethanol industry[15]. Searching for *Saccharomyces cerevisiae* that can tolerate low pH, ethanol efficiency, high temperature, and osmotic pressure in industrial ethanol production is one of the top research priorities[10,13,16].

Yeasts capacity to withstand several stressors has significant promise for application in large-scale fermentation.[10,17] Yeast isolates with a combination tolerance to various stress conditions in *vivo* have been found in earlier researcher.[13,18,19] However, the characterization of stress-tolerant yeast strains from local drinks for industrial bioethanol production is still insufficient. Obtaining yeast strains with high stress tolerance is currently one of the biggest challenges in producing bioethanol. This study intends to find potential multi-stress-tolerant yeasts that could be useful as a starter culture and an alternative to commercial strains of yeast. This study aimed to maximize the amount of produced bioethanol from sugar beet juice using stress-tolerant yeast strains under optimum conditions.

According to their capacity to withstand many stresses, four strains of yeast specifically, *S. cerevisiae* strains were chosen from the NCBI with their accession numbers, namely *Saccharomyces cerevisiae* isolate MUA1F (OR209275.1), *Saccharomyces cerevisiae* isolate MUA3F (OR209274.1), *Saccharomyces cerevisiae* isolate MUA11F (OR209277.1), and *Saccharomyces cerevisiae* isolate MUA15F (OR209276.1). Multi-stress tolerance yeasts were identified as *S. cerevisiae* strains after they were isolated and characterized in previous sections [20]. The four multi-stress-tolerant yeast strains that were chosen for this study can be useful in industrial bioethanol production.

Materials and methods

Raw material preparation

The study was carried out in the Microbiology Laboratory of the Department of Biology at Debre Markos University. Ten kg of fresh sugar beets were bought from a nearby supplier in Debre Markos town. The raw sugar beets were washed with clean water and cut into little pieces after being peeled. The pieces were crushed using a mechanical juicer to extract the juice. The extracted juice was filtered through muslin cloth to obtain raw juice concentrate and remove residual solids. The juice was preserved at 4°C to prevent any possible degradation during storage.

Sugar beet juice characterization

Raw sugar beet juice physicochemical characteristics including moisture content, pH, specific gravity, sucrose, and sulfated ash were estimated according were calculated using the method of [21,22]. The pH of the sugar beet juice was measured at room temperature using a digital pH meter (PHS-3C). A refractometer (Atagodensimeter model 2312) was used to test sugar beet juice Brix. The baume universal hydrometer was used to estimate the specific gravity. The Fehling method was used to calculate the amount of total sugar[23]. The 3, 5-dinitrosalicylic acid (DNS) method was used to evaluate the amount of reducing sugar[24]. The oven (MB45) drying method was used to estimate the amount of moisture present. The ash content was estimated using a muffle furnace set at 650°C for two hours until the weight remained constant.

Method of bioethanol production

Fermentation Medium Preparation

Sugar beet juice was diluted with distilled water to achieve the required Brix using a refractometer, and then 0.7 g L-1 ammonium sulfate was added to the medium and homogenized using a magnetic stirrer. Lastly, dilute sulfuric acid was used to bring the pH adjusted to 4.5, and the mixture was autoclaved for 15 minutes at 121°C. The sugar beet juice medium was utilized for fermentation and yeast strain multiplication in order to produce ethanol[25].

Yeast strain used in experiments

Saccharomyces cerevisiae isolate MUA1F (OR209275.1), MUA3F (OR209274.1), MUA11F (OR209277.1), and MUA15F (OR209276.1) with the accession numbers that had previously been isolated from *areke*[20]. Four native wild yeast strains were evaluated for their capacity to ferment sugar beet juice due to their exceptional ability to withstand a variety of stresses (see Supplementary Figure S1). Control was used as a standard methera industrial yeast strain for comparison with other strains.

Strains of yeast propagation

A loop that is full of fresh colonies taken from YEPD solid media and then inoculated into 100 ml of sterile glass flasks and treated with sugar beet juice. The glass flasks were left at 30°C for 24 hours. Each flask was then filled with 100 ml of fresh, sterilized sugar beet juice, and the flasks were cultured for an additional 24 hours to boost the number of cells (see Supplementary Figure S4). A 48-hour-propagated yeast culture was prepared as an inoculum for the fermentation of ethanol in a sugar beet juice media of 200 ml[26].

Optimizing the parameters of the fermentation process

Concentration of sugar beet juice (10, 15, 20, and 25 °Brix), temperature (25, 30, 35, and 40°C), pH (3.5, 4.0, 4.5, 5.0, and 5.5), ammonium sulfate addition (1, 2, 3, 4, and 5 gL-1), different supplements (urea, ammonium sulfate, yeast extract and ammonium nitrate), and incubation durations (24, 48, 72, 96, and 120 hours) were all optimized using the fed-batch fermentation method[27–29]. The method of one factor at a time (VAT) was applied. Inoculation with 48 hours of propagated yeast cells was performed in 800 milliliter bottles that contained 300 milliliters of sterilized diluted sugar beet juice. Every strain of yeasts inoculum was done by growing in 200 ml of sugar beet juice media for 48 hours at 30°C. Sterilized cotton was used to plug the inoculation bottles, and they were then incubated for 72 hours. After 72 hours, the samples were taken out, and the ethanol amount was measured. Each treatment was analyzed in triplicate, and the mean and standard error were calculated.

Fermentation under optimum conditions

The effect of varying concentrations of sugar beet juice (10, 15, 20, and 25°Brix) on ethanol concentration was evaluated at the optimum temperature of 30°C and 4.5 pH after 72 hours in fed-batch culture. Each of the strains of yeast was cultured at varying incubation temperatures (25, 30, 35, and

40°C) for ethanol production and was examined at the optimum 20 °Brix and 4.5 pH after 72 hours. The effect of various pH values (3.5, 4.0, 4.5, 5.0, and 5.5) on the strains of yeast to ethanol production was assessed at the optimum temperature of 30°C, 20 ^{O}Brix, after 72 hours. Each yeast strain's response to ammonium sulfate concentration (1, 2, 3, 4, and 5 g/l) in terms of production of ethanol was assessed at the optimum 30°C, 20 ^{O}Brix, pH 4.5, after 72 hours. The effects of the addition of supplements (1g/l of yeast extract, urea, and ammonium nitrate) on each strain's capacity to produce ethanol were also investigated at the optimum 30°C, 20 ^{O}Brix, pH 4.5 after 72 hours. The strains of yeast were cultivated in varying periods of fermentation (24, 48, 72, 96, and 120 hours) at optimum parameters 30°C, 20 ^{O}Brix, and pH 4.5. The ethanol concentration percentage of each fermentation parameter was calculated at the end of the fermentation time.

Analytical methods

A refractometer (Atagodensimeter model 2312) was used to measure the fermented samples Brix. UV-Vis Spectrometer (Spectrum Lab. 725s, India) set to 600 nm was utilized to figure out the propagating culture optical density[30]. A universal baume hydrometer was used to test the specific gravity of the fermented sugar beet juice to calculate its ethanol content[31]. The Martin,[32] the equation was used to calculate the ethanol concentration (ABV%)(see Supplementary Figure S3).

$$ABV = (SGin - SG) \times 131.25 \quad (1)$$

Where the initial specific gravity of the liquid before yeast is added is denoted by SGin, SG is the fermenting liquid's current specific gravity, and ABV is the alcohol by volume (%).

Data Analysis

Version 25.0 of the Statistical Package for Social Sciences software was employed in the analysis of all the data. The analysis of variance (ANOVA) was used to determine the means and standard deviations of the triplicate tests, and the post hoc test was used to see whether there were significant variations in the means ($p < 0.05$). The difference was determined to be statistically significant if $P < 0.05$ was less than 0.05.

Results and discussion

The physicochemical properties of sugar beet juice from a local supplier in Debre Markos town were composed of 13.21 ± 1.84% sugar, 12.26 ± 0.44% sucrose, and 0.41 ± 0.79% sulfated ash (w/v). The quantity of reducing sugar was 0.96 ± 0.12%. The moisture content of the sugar beet juice was 76.30 ± 0.26%. Moreover, the pH of the sugar beet juice was 5.53 ± 0.08. The findings matched with the results of [33,34].

The effect of the concentration of sugar beet juice

The effects of varying sugar beet juice concentrations (10%, 15%, 20%, and 25% Brix) on the percentage V/V of ethanol were investigated (Table 1; see Supplementary Figure S2). Among the factors that typically influence the rate at which yeasts produce ethanol is the amount of sugar present in the fermentation medium[35]. According to the current investigation, the four *areke* yeast strains produced a range of ethanol concentrations from 7.43 ± 0.17 to 11.77 ± 0.10% (v/v) of the amount in Brix of sugar beet juice between 10 and 25 (^{o}Brix). The Methera industrial yeast strain served as the control to compare it with different strains. The findings indicated that a sugar concentration of 20 °Brix was optimal for the strain and produced maximum ethanol concentrations of *Saccharomyces cerevisiae* isolate MUA15F (11.77 ± 0.10% (v/v) and *Saccharomyces cerevisiae* isolate MUA3F (11.71 ± 0.15% (v/v) at 72 hours, respectively. The quantity of ethanol in this investigation was consistent with the findings of other researchers[36,37]. At high concentrations of sugar beet juice (25%) all yeasts ethanol production dramatically reduced. This may be due to high substrate concentrations being inhibitory to the fermentation process of yeasts due to osmotic stress.

Table 1
Effects of varying sugar beet juice concentrations on the percentage V/V of ethanol in a fed-batch culture at 4.5 pH and 30°C after 72 hours.

	Molasses concentration (^{o}Brix)			
Yeast strain	10 ^{o}Brix	15 ^{o}Brix	20 ^{o}Brix	25 ^{o}Brix
Saccharomyces cerevisiae isolate MUA1F	7.43 ± 0.17^{bca}	9.04 ± 0.14^{abc}	9.97 ± 0.10^{ab}	9.43 ± 0.23^{ab}
Saccharomyces cerevisiae isolate MUA3F	8.54 ± 0.06^{b}	10.43 ± 0.18^{a}	11.71 ± 0.15^{a}	11.05 ± 0.08^{a}
Saccharomyces cerevisiae isolate MUA11F	8.37 ± 0.02^{bca}	9.75 ± 0.04^{abc}	10.84 ± 0.04^{ab}	10.34 ± 0.08^{ab}
Saccharomyces cerevisiae isolate MUA15F	8.55 ± 0.50^{c}	10.45 ± 0.12^{b}	11.77 ± 0.10^{b}	11.25 ± 0.50^{b}
Methara yeast standard	8.33 ± 0.11^{a}	10.47 ± 0.03^{c}	11.15 ± 0.04^{ab}	10.55 ± 0.12^{ab}

The means in the column that were followed by the same letter did not differ significantly at $P < 0.05$ using post hoc tests.

Effect of fermentation temperature

Temperature significantly affects on the fermenting organisms ability to develop, metabolize, and produce ethanol[23,35,38]. Different ethanol production results at various temperatures ranging from 25, 30, 35, and 40°C are displayed in Table 2. The production of ethanol gradually increases in parallel with the temperature increase and reaches the highest production for a temperature equal to 30°C. All the strains

were more effective at 30°C and produced less ethanol below and above 30°C (Table 2). In this finding, the highest quantity of ethanol was produced by each of the strains of yeast when the temperature reached 30°C. Ethanol produced by *Saccharomyces cerevisiae* isolate MUA3F (11.16 ± 0.08% (v/v) were showed statistically ($p \leq 0.05$) maximum concentration than all the remaining strains at 30°C. The findings matched with the results of [38–40]. All yeast strains produced substantially less ethanol at temperatures over 30°C or below 25°C because the enzyme activities are suppressed at low temperatures and denature at high temperatures.

Table 2
Effect of varying incubation temperature in fed-batch culture on ethanol concentration (%V/V) at 20 ^{o}Brix, 4.5 pH after 72 hours.

	Incubation Temperature			
Yeast strain	25°C	30°C	35°C	40°C
Saccharomyces cerevisiae isolate MUA1F	8.07 ± 0.10^{ab}	9.86 ± 0.14^{c}	9.01 ± 0.05^{bc}	8.53 ± 0.28^{ac}
Saccharomyces cerevisiae isolate MUA3F	9.44 ± 0.06^{ab}	11.16 ± 0.08^{b}	10.503 ± 0.10^{abd}	9.81 ± 0.10^{abd}
Saccharomyces cerevisiae isolate MUA11F	8.63 ± 0.13^{a}	10.33 ± 0.08^{a}	9.01 ± 0.29^{a}	8.63 ± 0.07^{b}
Saccharomyces cerevisiae isolate MUA15F	9.08 ± 0.02^{ab}	10.54 ± 0.05^{ab}	10.08 ± 0.03^{acd}	9.31 ± 0.20^{bcd}
Methara yeast standard	8.74 ± 0.08^{b}	10.54 ± 0.12^{ac}	9.44 ± 0.03^{d}	8.53 ± 0.06^{d}

The means in the column that were followed by the same letter did not differ significantly at $P < 0.05$ using post hoc tests.

Effect of pH

Optimum pH levels are necessary for plasma membrane-based proteins and enzymes to function[41–43]. The results of the study on the effect of pH ranges between 3.5 to 5.5 on the production of ethanol are shown in Table 3. According to the current study, the production of ethanol gradually increases along with pH increase and achieved greatest production at pH equal to 4.5, and then it started to decline. *Saccharomyces cerevisiae* isolate MUA3F (11.05 ± 0.08% (v/v) were showed statistically ($P \leq 0.05$) higher production of ethanol than the other strains. Numerous researchers noticed that *S. cerevisiae* optimum pH was 4.5[38,40,44,45]. The *Saccharomyces cerevisiae* isolate MUA1F had the minimum content of ethanol (10.14 ± 0.24% (v/v)) at pH 4.5, with all other strains having statistically significant values ($P \leq 0.05$). In contrast to the finding, the pH range of 4.0 to 5.0 is optimum for alcohol production by *S. cerevisiae*[9].

Table 3

The effect of varying pH in fed-batch culture on ethanol concentration (%V/V) at 30°C, 20 ^{o}Brix, after 72 hours.

	pH				
Yeast strain	pH 3.5	pH 4	pH 4.5	pH 5	pH 5.5
Saccharomyces cerevisiae isolate MUA1F	6.08 ± 0.22^{bde}	8.06 ± 0.10^{ab}	10.14 ± 0.24^{ad}	9.20 ± 0.05^{bd}	8.14 ± 0.12^{b}
Saccharomyces cerevisiae isolate MUA3F	6.53 ± 0.06^{b}	8.77 ± 0.10^{ac}	11.05 ± 0.08^{ac}	10.51 ± 0.29^{abc}	9.92 ± 0.07^{ab}
Saccharomyces cerevisiae isolate MUA11F	6.22 ± 0.11^{ca}	8.44 ± 0.05^{abc}	10.34 ± 0.08^{cd}	9.41 ± 0.03^{a}	8.15 ± 0.11^{a}
Saccharomyces cerevisiae isolate MUA15F	6.51 ± 0.07^{de}	8.54 ± 0.04^{bd}	10.90 ± 0.09^{bdf}	10.04 ± 0.10^{ad}	9.35 ± 0.30^{ab}
Methara yeast standard	6.91 ± 0.18^{ae}	8.14 ± 0.06^{cd}	10.54 ± 0.12^{c}	9.55 ± 0.12^{c}	8.88 ± 0.26^{ab}

The means in the column that were followed by the same letter did not differ significantly at $P < 0.05$ using post hoc tests.

The effect of supplement concentration

Adequate nitrogen is frequently included in sugar beet juice to encourage ethanol production and yeast growth[46–48]. Numerous experiments were conducted in the past to optimize nitrogen sources and other supplements[48–50]. The data gathered showed that ethanol production increased with ammonium sulfate addition to the fermentation medium between 1.0 and 4.0 g/l. At their optimum conditions, an ammonium sulfate concentration of 4.0 g/l resulted in higher ethanol production. The current findings, the quantity of ethanol produced by *Saccharomyces cerevisiae* isolate MUA3F increased from 11.28 ± 0.08 to 12.49 ± 0.22% (v/v) between 1.0 to 4.0 g/l ammonium sulfate. According to Darvishi *et al.*[51] the optimal medium for *S. cerevisiae* to make ethanol was ammonium sulfate (4 g/L) with 10% (v/v) ethanol content.

Table 4
Ammonium sulphate (NH_4SO_4) concentrations effect on the amount of ethanol (V/V) in a fed batch culture at 30°C, 20 ^{o}Brix, pH 4.5, and 72 hours.

	Ammonium sulphate (NH_4SO_4) concentration				
Yeast strain	1g NH_4SO_4	2g NH_4SO_4	3g NH_4SO_4	4g NH_4SO_4	5g NH_4SO_4
Saccharomyces cerevisiae isolate MUA1F	9.90 ± 0.05^{b}	10.32 ± 0.30^{cd}	10.65 ± 0.06^{bc}	10.98 ± 0.01^{abc}	10.69 ± 0.35^{ac}
Saccharomyces cerevisiae isolate MUA3F	11.28 ± 0.08^{ab}	11.45 ± 0.16^{abc}	11.89 ± 0.07^{ab}	12.49 ± 0.22^{ac}	12.00 ± 0.12ab
Saccharomyces cerevisiae isolate MUA11F	10.25 ± 0.08^{b}	10.46 ± 0.04^{b}	10.78 ± 0.05^{a}	11.43 ± 0.21^{ae}	10.79 ± 0.03^{b}
Saccharomyces cerevisiae isolate MUA15F	10.52 ± 0.02^{ba}	10.97 ± 0.17^{abd}	11.44 ± 0.10^{abc}	11.90 ± 0.20^{a}	11.75 ± 0.10^{bc}
Methara yeast standard	10.17 ± 0.12^{a}	10.28 ± 0.22^{a}	10.74 ± 0.03^{b}	12.23 ± 0.12^{be}	11.35 ± 0.06^{bc}

The means in the column that were followed by the same letter did not differ significantly at $P < 0.05$ using post hoc tests.

Effect of different supplements

At a 1 g supplementation/l concentration, the effects of several nitrogen sources (yeast extract, urea, and ammonium nitrate) were studied in Table 5. Compared to other sources of nitrogen and the control, yeast extract supplementation produced significant levels of ethanol. Urea was the second nitrogen source, following ammonium nitrate that enhanced ethanol production. In contrast to the other supplemented medium, the medium containing ammonium nitrate had the lowest amount of ethanol. *Saccharomyces cerevisiae* isolate MUA15F was produced the maximum amount of ethanol from the nitrogen source yeast extract (11.87 ± 0.07% (v/v). These results confirmed Abdel-Hamied *et al.*[45], who showed that the ethanol content increased significantly upon the addition of yeast extracts. Convenient growth ingredients for yeast growth are provided by yeast extract[52,53]. The ethanol content was lowest in all strains of yeast cultivated without sources of nitrogen (control).

Table 5

The effect of various supplements on the percentage V/V of ethanol in a fed batch culture at 20 ^{o}Brix, pH 4.5, 30°C, and 4.5 pH after 72 hours.

	Different nitrogen sources			
Yeast strain	Yeast Extract	Urea	NH_4NO_3	Control
Saccharomyces cerevisiae isolate MUA1F	10.33 ± 0.10^{a}	10.05 ± 0.03^{ce}	9.77 ± 0.02^{cb}	9.50 ± 0.10^{cf}
Saccharomyces cerevisiae isolate MUA3F	11.61 ± 0.06^{ab}	11.15 ± 0.06^{bcd}	10.45 ± 0.23^{acd}	9.88 ± 0.10^{ac}
Saccharomyces cerevisiae isolate MUA11F	10.44 ± 0.12^{bc}	10.16 ± 0.06^{ab}	10.12 ± 0.02^{a}	9.41 ± 0.07^{a}
Saccharomyces cerevisiae isolate MUA15F	11.87 ± 0.07^{ac}	11.00 ± 0.21^{aef}	10.21 ± 0.06^{bf}	9.65 ± 0.15^{bd}
Methara standard yeast	10.86 ± 0.12^{ac}	10.35 ± 0.08^{df}	9.78 ± 0.04^{df}	8.48 ± 0.12^{adf}

The means in the column that were followed by the same letter did not differ significantly at $P < 0.05$ using post hoc tests.

The effect of incubation times

Microorganism growth affects the length of time fermentation takes, and a shorter fermentation period results in ineffective fermentation because of insufficient microbe growth[54,55]. The effect of incubation durations on production of ethanol after fermentation were conducted for 24, 48, 72, 96, and 120 hours is seen in Table 6. During the 72 hours incubation phase, ethanol production increased; however, it then dropped again to the lowest levels at the 120 hours. The highest production of ethanol was showed at the 72 hours of fermentation. The research revealed that the strain *Saccharomyces cerevisiae* isolate MUA3F achieved its maximum ethanol production after 72 hours, with a concentration of 11.15 ± 0.08% (v/v). It was accepted that after three days of growth, yeasts can ferment sugary substrates to produce the maximum ethanol amounts.[56,57] The ethanol production increased gradually, but as the incubation period increased, the ethanol concentration somewhat decreased. This could be because the yeast cells were no longer able to survive and consume the ethanol[55,58]. The present findings supported the findings of previous studies showing that the optimization of sugar beet juice fermentation parameters, including temperature, pH, time, and substrate concentration, is crucial for maximizing ethanol production[56,59].

Table 6
The effect of various incubation times on the percentage V/V of ethanol in a fed batch culture at 20 ^{o}Brix, pH 4.5, 30°C, and pH 4.5 after 72 hours.

	Fermentation Time				
Yeast strain	**24 hours**	**48 hours**	**72 hours**	**96 hours**	**120 hours**
Saccharomyces cerevisiae isolate MUA1F	7.08 ± 0.02^{bc}	8.90 ± 0.50^{c}	9.40 ± 0.30^{c}	9.09 ± 0.08^{bcd}	8.70 ± 0.09^{bd}
Saccharomyces cerevisiae isolate MUA3F	7.89 ± 0.03^{b}	8.91 ± 0.05^{b}	11.14 ± 0.08^{b}	10.91 ± 0.03^{abd}	10.15 ± 0.16^{abc}
Saccharomyces cerevisiae isolate MUA11F	8.91 ± 0.03^{ab}	9.44 ± 0.12^{acd}	10.34 ± 0.08^{a}	9.13 ± 0.30^{ad}	8.61 ± 0.03^{a}
Saccharomyces cerevisiae isolate MUA15F	7.87 ± 0.19^{acd}	8.76 ± 0.06^{a}	10.15 ± 0.16^{ac}	9.98 ± 0.08^{ac}	9.09 ± 0.16^{adf}
Methara standard yeast	7.64 ± 0.09^{bd}	8.77 ± 0.17^{d}	10.54 ± 0.12^{abc}	9.83 ± 0.16^{d}	8.61 0.10^{cf}

The means in the column that were followed by the same letter did not differ significantly at $P < 0.05$ using post hoc tests.

Conclusion

The highest concentration of ethanol produced by *Saccharomyces cerevisiae* isolate MUA3F was 12.49 ± 0.22% (v/v) at 30°C, 20 °Brix sugar beet juice, 4g NH_4SO_4, 4.5 pH, and 72 hours fermentation. Under optimal fermentation conditions, the candidate yeast strains (*Saccharomyces cerevisiae* isolates MUA3F and MUA15F) produced the highest levels of alcohol and may be utilized in bioethanol production. Therefore, a local beverage called *areke* may be a good source of yeasts that can produce high amounts of bioethanol.

Declarations

Competing interests

The author declares no competing interests.

Ethical approval

Not applicable

Funding Statement

This research was supported by Debre Markos University.

Author Contribution

Conceptualization, M.F; Methodology, M.F; Data analysis, M.F; Investigation, M.F; Resources, M.F; Writing–original draft, M.F; Writing–review and editing, M.F; Visualization, M.F; Funding acquisition, M.F. The author has read and agreed to the publication of the manuscript.

Acknowledgement

I would like to thank Methara Bioethanol Factory for providing the standard yeast strain.

Data Availability

Data will be made available on request. The yeast strain used in experiments are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/genbank/), with the GenBank accession number of Saccharomyces cerevisiae isolate MUA1F (OR209275.1), MUA3F (OR209274.1), MUA11F (OR209277.1), and MUA15F (OR209276.1).

References

1. Malaquias, A. C. T. *et al.* The misleading total replacement of internal combustion engines by electric motors and a study of the Brazilian ethanol importance for the sustainable future of mobility: a review. *J Braz Soc Mech Sci Eng.* 41, 1–23 (2019).
2. Simonyan, K. J. & Fasina, O. Biomass resources and bioenergy potentials in Nigeria. *African J. Agric. Res.* **8**, 4975–4989 (2013).
3. Sombilla, M. A., Malik, U. S., Ahmed, M. A. K. & Cueno, S. L. *Integrating Biofuel and Rural Renewable Energy Production in Agriculture for Poverty Reduction in the Greater Mekong Subregion: An Overview and Strategic Framework for Biofuel Development:* (2009).
4. Scott, A. & Bryner, M. Alternative fuels: Rolling out next-generation technologies. *Chem. Week.* **27**, 17-21 (2006).

5. Dodic, S. *et al.* Bioethanol production from thick juice as intermediate of sugar beet processing. *Biomass and Bioenergy*. **33**, 822–827 (2009).
6. Koga, N. An energy balance under a conventional crop rotation system in northern Japan: Perspectives on fuel ethanol production from sugar beet. *Agric. Ecosyst. Environ.* **125**, 101–110 (2008).
7. Cardenas-Fernandez, M. *et al.* An integrated biorefinery concept for conversion of sugar beet pulp into value-added chemicals and pharmaceutical intermediates. *Faraday Discuss.* **202**, 415–431 (2017).
8. De Oliveira, R. A. *et al.* A simple biorefinery concept to produce 2g-lactic acid from Sugar Beet Pulp (SBP): A high-value target approach to valorize awaste stream. *Molecules*. **25**, (2020).
9. Lin, Y. *et al.* Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. *Biomass and Bioenergy*. **47**, 395–401 (2014).
10. Andrietta, M. G. S., Andrietta, S. R., Steckelberg, C. & Stupiello, E. N. A. Bioethanol - Brazil, 30 years of Proálcool. *Int. Sugar J.* **109**, 195–200 (2007).
11. Abdel-Banat, B. M. A., Hoshida, H., Ano, A., Nonklang, S. & Akada, R. High-temperature fermentation: How can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Appl. Microbiol. Biotechnol.* **85**, 861–867 (2010).
12. Fleet, G. H. Wine yeasts for the future. *FEMS Yeast Res.* **8**, 979–995 (2008).
13. Tesfaw, A., Oner, E. T. & Assefa, F. Optimization of ethanol production using newly isolated ethanologenic yeasts. *Biochem. Biophys. Reports*. **25**, 100886 (2021).
14. Talukder, A. A., Easmin, F., Mahmud, S. A. & Yamada, M. Thermotolerant yeasts capable of producing bioethanol: isolation from natural fermented sources, identification and characterization. *Biotechnol. Biotechnol. Equip.* **30**, 461- 475 (2016).
15. Radecka, D. *et al.* Looking beyond *Saccharomyces*: The potential of non-conventional yeast species for desirable traits in bioethanol fermentation. *FEMS Yeast Research*. **15**, 53 (2015).
16. Guyot, S., Ferret, E. & Gervais, P. Responses of *Saccharomyces cerevisiae* to thermal stress. *Biotechnol. Bioeng.* **92**, 403–409 (2005).
17. Snowdon, C., Schierholtz, R., Poliszczuk, P., Hughes, S. & Van Der Merwe, G. ETP1/YHL010c is a novel gene needed for the adaptation of *Saccharomyces cerevisiae* to ethanol. *FEMS Yeast Res.* **9**, 372–380 (2009).
18. Andualem, B., Shiferaw, M. & Berhane, N. Isolation and characterization of *Saccaromyces cervisiae* yeasts isolates from “tella” for beer production. *Annu. Res. Rev. Biol.* **15**, 1–12 (2017).
19. Teramoto, Y., Sato, R. & Ueda, S. Characteristics of fermentation yeast isolated from traditional Ethiopian honey wine, ogol. *African J. Biotechnol.* **4**, 160–163 (2005).
20. Fentahun, M., & Andualem, B. Characterization of wild indigenous yeasts that can withstand different stresses from traditional fermented beveragesin the Amhara Region, Ethiopia. *Food Sci. Appl. Biotechnol.***7**, 262- 277 (2024).

21. Official methods of analysis of AOAC International WorldCat.org. https://www.worldcat.org/title/official-methods-of-analysis-of-aoac-international/oclc/44761301 (accessed 30 December 2022).
22. ICUMSA - Verlag Dr. Albert Bartens KG, https://sugarindustry.info/icumsa/ (accessed 28 December 2022).
23. Periyasamy, S., Venkatachalam, S., Ramasamy, S. & Srinivasan, V. Production of Bio-ethanol from Sugar Molasses Using *Saccharomyces Cerevisiae*. *Mod. Appl. Sci.* **3**, 32 (2009).
24. Deshavath, N. N., Mukherjee, G., Goud, V. V., Veeranki, V. D. & Sastri, C. V. Pitfalls in the 3, 5-dinitrosalicylic acid (DNS) assay for the reducing sugars: Interference of furfural and 5-hydroxymethylfurfural. *Int. J. Biol. Macromol.* **156**, 180- 185 (2020).
25. Dziugan, P., Balcerek, M., Pielech-Przybylska, K. & Patelski, P. Evaluation of the fermentation of high gravity thick sugar beet juice worts for efficient bioethanol production. *Biotechnol. Biofuels.* **6**, 2–11 (2013).
26. Willey, J. M., Sandman, K. M. & Wood, D. H. *Prescott' s Microbiology*.11th edn. McGraw–Hill companies, New York, **41**, 964–976 (2002).
27. Fadel, M., Keera, A. A., Mouafi, F. E. & Kahil, T. High Level Ethanol from Sugar Cane Molasses by a New Thermotolerant *Saccharomyces cerevisiae* Strain in Industrial Scale. *Biotechnol. Res. Int.* **2013**, 1–6 (2013).
28. De Vasconcelos, J. N., Lopes, C. E. & De França, F. P. Continuous ethanol production using yeast immobilized on sugar-cane stalks. *Brazilian J. Chem. Eng.* **21**, 357–365 (2004).
29. Altınışık, S. *et al.* Optimization of bioethanol production from sugar beet processing by-product molasses using response surface methodology. *Biomass Convers. Biorefinery.* 9875–9888 (2024).
30. Brian, C. & Matlock. Differences in bacterial optical density measurements between UV-Visible spectrophotometers, *Thermo Fisher Scientific.* (2019).
31. Martin, S. The SAGE Encyclopedia of Alcohol: Social, Cultural, and Historical Perspectives. *SAGE Encycl. Alcohol Soc. Cult. Hist. Perspect.* C, (2015).
32. Martin, S. Alcohol by Volume. *SAGE Encycl. Alcohol Soc. Cult. Hist. Perspect.* **7** (2015)
33. Mahajan, S. & Singh Kocher, G. Standardization of sugar beet juice extraction by microwave heating and its fermentation for bioethanol production. *Int. J. Chem. Stud.* **6**, 2023–2027 (2018).
34. Popov, S., Rankovic, J., Dodic, J., Dodic, S. & Jokic, A. Bioethanol production from raw juice as intermediate of sugar beet processing: A response surface methodology approach. *Food Technol. Biotechnol.* **48**, 376–383 (2010).
35. Phisalaphong, M., Srirattana, N. & Tanthapanichakoon, W. Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation. *Biochem. Eng. J.* **28**, 36–43 (2006).
36. Kawa-Rygielska, J., Pietrzak, W., Regiec, P. & Stencel, P. Utilization of concentrate after membrane filtration of sugar beet thin juice for ethanol production. *Bioresour. Technol.* **133**, 134–141 (2013).

37. Ogbonna, J. C., Mashima, H. & Tanaka, H. Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor. *Bioresour. Technol.* **76**, 1–8 (2001).
38. Asif, H. K. *et al.* Comparative study of bioethanol production from sugarcane molasses by using *Zymomonas mobilis* and *Saccharomyces cerevisiae*. *African J. Biotechnol.* **14**, 2455–2462 (2015).
39. Tan, L. *et al.* Production of ethanol from raw juice and thick juice of sugar beet by continuous ethanol fermentation with flocculating yeast strain KF-7. *Biomass and Bioenergy* **81**, 265–272 (2015).
40. Zaghlou, R. A., Ismail, S. A., Enan, G., El-Meihy, R. M. & Abdel-Rahman, H. M. Maximization of Bio-Ethanol Production by Yeasts using Sugar Cane and Sugar Beet Molasses. *Adv. Anim. Vet. Sci.* **9**, 2069–2076 (2021).
41. Kasemets, K., Nisamedtinov, I., Laht, T. M., Abner, K. & Paalme, T. Growth characteristics of *Saccharomyces cerevisiae* S288C in changing environmental conditions: Auxo-accelerostat study. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* **92**, 109–128 (2007).
42. Arroyo-López, F. N., Orlic, S., Querol, A. & Barrio, E. Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid. *Int. J. Food Microbiol.* **131**, 120–127 (2009).
43. Reda, F. M., Hussein, B. M. & Enan, G. Selection and characterization of two probiotic lactic acid bacteria strains to be used as starter and protective cultures for food fermentations. *J. Pure Appl. Microbiol.* **12**, 1499–1513 (2018).
44. Hemamalini, Hema, C. & Geetha, S. Comparative study of continuous ethanol fermentation from molasses by using *Saccharomyces cervisiae* and *Schizosaccharomyces pombe*. **1**, 219-228 (2012).
45. Rasmey, A.-H. M., Hassan, H. H., Abdulwahid, O. A. & Aboseidah, A. A. Enhancing Bioethanol Production from Sugarcane Molasses by *Saccharomyces cerevisiae* Y17. *Egypt. J. Bot.* **58**, 547–561 (2018).
46. Mendes-Ferreira, A., Mendes-Faia, A. & Leão, C. Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry. *J. Appl. Microbiol.* **97**, 540–545 (2004).
47. Nofemele, Z., Shukla, P., Trussler, A., Permaul, K. & Singh, S. Improvement of ethanol production from sugarcane molasses through enhanced nutrient supplementation using *Saccharomyces cerevisiae*. *J. Brew. Distill.* **3**, 29–35 (2012).
48. Pretorius, I. S., Du Toit, M. & Van Rensburg, P. Designer yeasts for the fermentation industry of the 21st century. *Food Technol. Biotechnol.* **41**, 3–10 (2003).
49. Malherbe, S., Bauer, F. F. & Du Toit, M. Understanding problem fermentations - A review. *South African J. Enol. Vitic.* **28**, 169–186 (2007).
50. Cazetta, M. L., Celligoi, M. A. P. C., Buzato, J. B. & Scarmino, I. S. Fermentation of molasses by Zymomonas mobilis: effects of temperature and sugar concentration on ethanol production. *Bioresour. Technol.* **98**, 2824–2828 (2007).

51. Darvishi, F. & Moghaddami, N. A. Optimization of an Industrial Medium from Molasses for Bioethanol Production Using the Taguchi Statistical Experimental-Design Method. *Ferment,***5**, 14 (2019).
52. Nancib, N. *et al.* The effect of supplementation by different nitrogen sources on the production of lactic acid from date juice by *Lactobacillus casei subsp. rhamnosus. Bioresour. Technol.* **78**, 149–153 (2001).
53. Ortiz-Muniz, B., Carvajal-Zarrabal, O., Torrestiana-Sanchez, B. & Aguilar-Uscanga, M. G. Kinetic study on ethanol production using *Saccharomyces cerevisiae* ITV-01 yeast isolated from sugar cane molasses. *J. Chem. Technol. Biotechnol.* **85**, 1361–1367 (2010).
54. Tofighi, A., Mazaheri Assadi, M., Asadirad, M. H. A. & Karizi, S. Z. Bio-ethanol production by a novel autochthonous thermo-tolerant yeast isolated from wastewater. *J. Environ. Heal. Sci. Eng.* **12**, (2014).
55. Zabed, H. *et al.* Bioethanol production from fermentable sugar juice. *Sci. World J.* (2014).
56. Shaghaghi-Moghaddam, R., Jafarizadeh-Malmiri, H., Mehdikhani, P., Alijanianzadeh, R. & Jalalian, S. Optimization of submerged fermentation conditions to overproduce bioethanol using two industrial and traditional *Saccharomyces cerevisiae* strains. *Green Process. Synth.* **8**, 157–162 (2019).
57. Van Maris, A. J. A. *et al.* Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *A. van Leeuwenhoek.* **90**, 391–418 (2006).
58. De La Cruz, V., Hernandez, S., Martin, M. & Grossmann, I. E. Integrated Synthesis of Biodiesel, Bioethanol, Isobutene, and Glycerol Ethers from Algae. *Ind. Eng. Chem. Res.* **53**, 14397–14407 (2014).
59. Jambo, S. A., Abdulla, R., Marbawi, H. & Gansau, J. A. Response surface optimization of bioethanol production from third generation feedstock - Eucheuma cottonii. *Renew. Energy* **132**, 1–10 (2019).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- editablefigSupplementalmaterialSReports.pdf