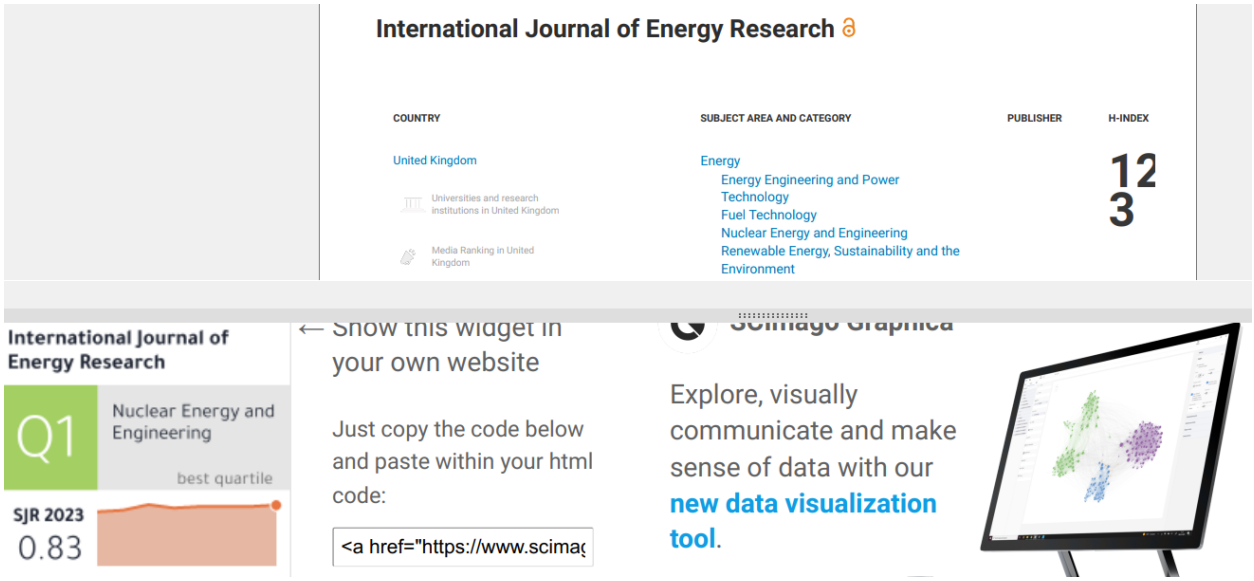

Review invitation==International Journal of Energy Research

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Astrilia Damayanti <astrilia.damayanti@mail.unnes.ac.id>

7086047: Review invitation
1 message

International Journal of Energy Research <ijer@hindawi.com> Thu, Feb 15, 2024 at 3:26 PM
Reply-To: International Journal of Energy Research <sharon.habacon@hindawi.com>
To: Astrilia Damayanti <astrilia.damayanti@mail.unnes.ac.id>

WILEY

Dear Dr. Damayanti,

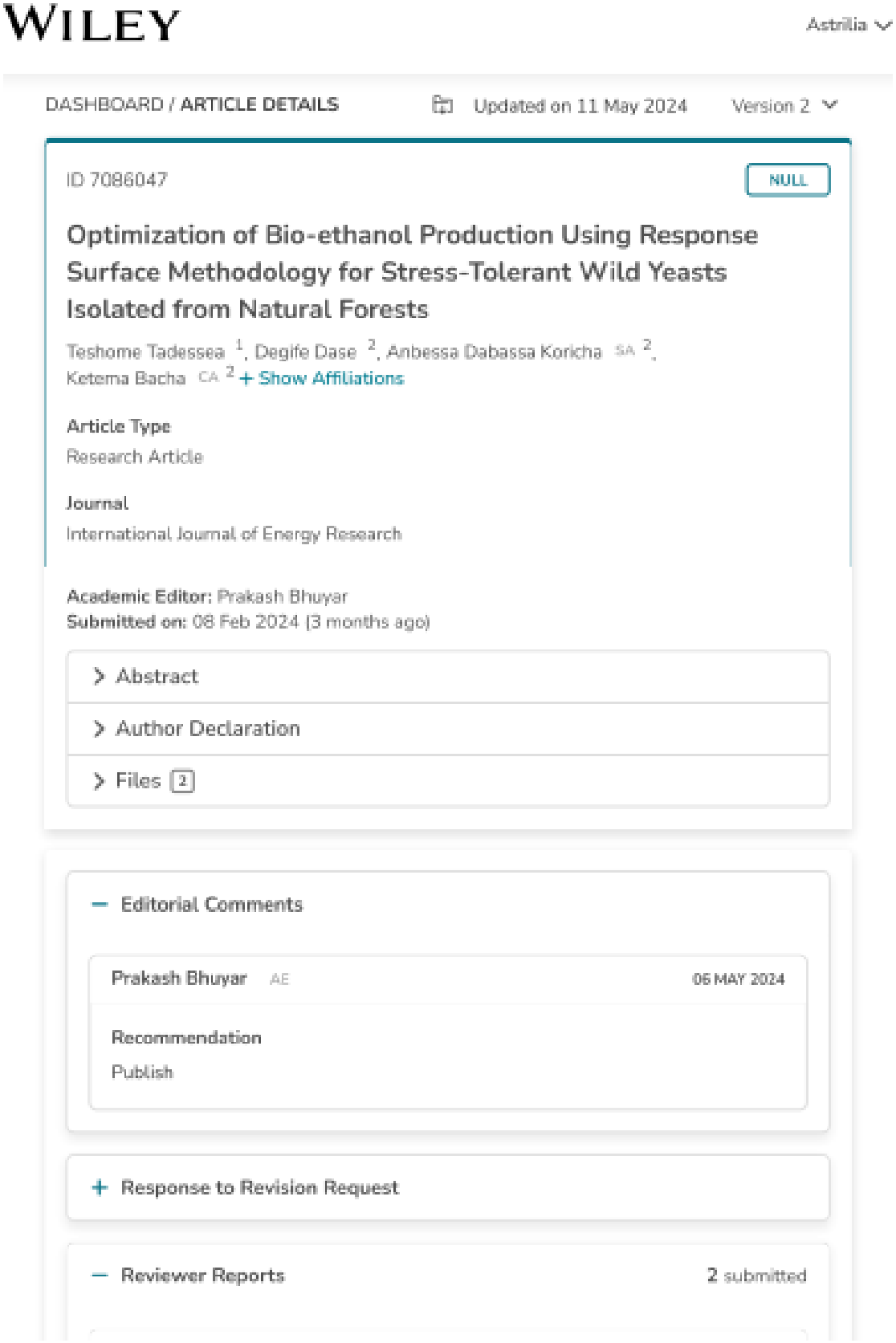
A manuscript titled "Optimization of Bio-ethanol Production Using Response Surface Methodology for Stress-Tolerant Wild Yeasts Isolated from Natural Forests" by Anbessa Dabassa Koricha, has been submitted to International Journal of Energy Research for consideration.

As the Academic Editor handling the manuscript, I would be delighted if you would agree to review it and let me know whether you feel it is suitable for publication.

DECLINE

AGREE

Reviewer Reports



Reviewer 2: 06 May 2024

REVIEWER ACTIVITY

Invited: 28 Apr 2024

Accepted: 29 Apr 2024

Submitted: 06 May 2024

Recommendation: PUBLISH

Reviewer Report: The authors respond clearly to all comments and suggestions made.

Reviewer 1: Astrilia Damayanti 06 May 2024

Affiliation: Universitas Negeri Semarang

REVIEWER ACTIVITY

Invited: 28 Apr 2024

Accepted: 28 Apr 2024

Submitted: 06 May 2024

Recommendation: PUBLISH

Reviewer Report: all corrections have been corrected in the manuscript.

Response to Reviewer's Comment (R#1)

(1) Response to Reviewer's Comment (R#1)

This paper described the ethanol production of wild yeasts isolated from natural forests. The optimization condition for ethanol production by the selected yeast isolate was also performed using RSM based on CCD. The obtained results are interesting. However, several points must be proved and corrected to improve this manuscript's quality and novelty.

Abstract:

(**Q1**). The authors mentioned that *S. cerevisiae* 9Li2 was able to ferment 40 g/L glucose to produce 15 g/L ethanol at 40 °C with 73% efficiency. I wonder which part of the experiment this result comes from because most of the experiments for ethanol production were carried out at 30 °C. The optimal conditions for ethanol production predicted based on the statistical model also highlighted the optimum temperature at around 30 °C, not 40 °C.

- Included

1. Ethanol fermentation using only 40 g/L of glucose is not extractive for industrial production since most commercial ethanol production uses 200 to 220 g/L of initial sugar concentrations. What is the advantage of using 40 g/L glucose to produce ethanol?

- Energy Efficiency: Fermentation at lower sugar concentrations can be conducted at moderate temperatures (around 35°C to 40°C), which may require less energy compared to processes that need higher temperatures. That is why we use 40g/L for intial sugar concentaration to produce ethanol.
- For the initial sugar, we just utilized 40 g/L on a laboratory scale, and it worked quite well. Additionally, we employed a greater concentration for four particular yeasts and control, ranging from 20 to 220 g/L. Of the yeasts that were chosen, 9Li2 and 35L1 showed a noteworthy rise in ethanol production when the glucose content was raised from 40 to 220 g/L. Because the other 2 isolates did not produce ethanol above 120 g/L of glucose, we were only able to demonstrate glucose concentrations up to that point (120 g/L) until your comments were made.
- Based on the literature review, benefits of Very High Gravity (VHG) (200 to 210 g/L) ethanol fermentation include decreased water requirements, fewer chances of contamination, and lower distillation costs. However, a high ethanol-producing strain is required for effective fermentation because yeast cells must be able to withstand high osmotic pressure and ethanol concentration.

Introduction:

1. Based on the literature review, the statistical model, specifically RSM based on CCD, has been widely used to find the optimal conditions for ethanol production by yeasts isolated from different places. What is the novelty of this study if the authors focus on RSM?

- **The novelty of this manuscript are the following**:
- (1) New Yeast Strains: We used yeast strains that have not been previously characterized for ethanol production, the RSM can help in understanding the optimal conditions for these new strains.

- (2) Unique Substrates: Employing RSM to optimize ethanol production from non-traditional or locally available substrates can provide novel insights into the use of alternative resources.
- (3) Process Optimization: Even if RSM is a well-known method, its application to optimize a specific set of parameters (like temperature, pH, inoculum size, etc.) for a particular yeast strain can still yield unique, valuable data.

- Moreover, the main advantage of RSM is reduced number of experimental runs needed to provide sufficient information for statistically acceptable results, its suitability for multiple factor experiments and exploration of common relationship between various factors towards finding the most appropriate production conditions for the bioprocess and forecast response.

Materials and Methods:

1. For osmo-tolerance analysis, why did the authors use glucose in this experiment? Since yeast cells can metabolize glucose and produce ethanol during cultivation. The synergetic effect of high glucose and ethanol may occur. Using other substances that can not be metabolized by yeast cells instead of glucose is better.
   In their osmo-tolerance analysis, the authors chosen to use glucose for the following reasons. The reason why glucose was the preferred substrate is:

- **(1) Metabolism and Tolerance Assessment**:
  - Glucose is a readily metabolizable sugar that yeast cells can efficiently break down through glycolysis.
  - By using glucose, the researchers assessed how well yeast cells tolerate osmotic stress while metabolizing a common sugar.
  - Furthermore, the experiment aimed to understand how yeast cells respond to high osmolarity in the presence of a metabolizable substrate.
- **(2) Standardization and Comparisons**:
  - Glucose serves as a standard reference in many biochemical experiments due to its well-characterized metabolism.
  - Using glucose allows for direct comparisons with existing literature and other studies.
  - Researchers established a baseline for osmo-tolerance by measuring glucose utilization under varying conditions.
- **(3) Practical Considerations**:
  - Glucose is abundant and economically accessible.
  - Its availability simplifies experimental design and ensures reproducibility.
  - Other substances that yeast cells cannot metabolize might introduce confounding factors or obscure the specific effects of osmotic stress.
- **(4) Ethanol Production and Synergy**:

- While it's true that yeast cells can produce ethanol from glucose, this aspect is one relevant variable to the study.
- The synergetic effect of high glucose and ethanol could provide insights into how yeast cells cope with osmotic stress.
- By including glucose, the researchers explored potential interactions between osmotic stress, glucose metabolism, and ethanol production.

Therefore, glucose was chosen for its metabolic relevance, standardization, practicality, and the opportunity to study synergies with ethanol.

We have taken note of your feedback, however for the time being, glucose is our sole concern to produce ethanol in the presence of multiple stresses wild yeasts.

2. How did the authors test the effect of acetic acid on yeast growth? There is no information in the manuscript.
   Incuded
3. In the effect of glucose concentrations on ethanol production, why did the authors test sugar at a concentration of up to 120 g/L? The authors claim that the isolated yeast can withstand sugar up to 600 g/L (60%) (Table 2), so experiments using sugar concentrations higher than 120 g/L should be performed.
   The results of 140 to 220 g/l were included
4. It is better to perform the experiments on the effect of temperature on ethanol production since the authors claim that the isolated yeasts can withstand temperatures up to 45 °C.

- This is a topographical error that has been corrected in the document.
- *S. cerevisiae* 9Li2 produced a significant amount of ethanol (14.8768 g/L ~ 15 g/L) at the optimal temperature (40 °C), pH (5.167), and duration (72 hours). However, since the production of ethanol dropped as the temperature rose from 35 to 40 °C, we did not report the results above 40 °C.

5. Many factors affect ethanol production by yeasts; why do the authors focus only on temperature, pH, and fermentation time? There is a statistical model to screen the factors that affect ethanol production, like ***the Plackett–Burman*** design; why did the authors use this design to screen the factors?

The reason why the authors focused on Temperature, pH, and Fermentation Time is:

- **(1) Temperature**: Yeast metabolism is highly temperature-dependent. Optimal temperatures enhance enzyme activity and promote efficient fermentation. Too high or too low temperatures can negatively impact ethanol production.
- **(2) pH**: Yeast enzymes function optimally within specific pH ranges. Deviations from the optimal pH affect enzyme activity and overall fermentation efficiency.
- **(3) Fermentation Time**: Longer fermentation times allow yeasts to fully utilize available substrates, leading to higher ethanol yields. However, excessively long times may also lead to reduced productivity.

The reason why not the authors used Plackett–Burman model Design is:

- The Plackett–Burman design is a **screening experimental design** used to identify significant factors affecting a process.

However, the authors focused on temperature, pH, and fermentation time because these factors directly influence yeast metabolism and ethanol production. The Plackett–Burman design allowed them to efficiently screen factors and identify key variables for further investigation. This design is typically used when there are more than five independent variables, which is not the case in our study. Therefore, we recommend using this model design for future work.

- We maintained constants for inoculum size and substrate concentration and concentrated primarily on temperature, pH, and fermentation time. We aimed to produce large amounts of ethanol with high productivity and to investigate the thermotolerant capacity of multi-stressed wild yeasts to grow and ferment glucose at high temperatures. To enhance the performance of ethanol fermentation, the effects of temperature, pH level, length of fermentation, and initial glucose content on ethanol production by wild yeasts were assessed.
- We were not aware of the Plackett-Burman method as of yet. We certainly committed to using it in the upcoming study. Now we understood as it was recognized that Plackett-Burman designs could effectively screen a large number of factors in a small number of trials.

Results and discussion:

1. The data presented in Table 2 is not reliable. Viable cells or the growth profiles (like OD) are more valuable and convincing. Please change the data in Table 2.
   Changed

2. It is unclear how the isolated yeasts can withstand glucose concentrations up to 50% (500 g/L) or 60% (600 g/L). The growth profile or the yeast cell morphology should be presented to confirm this circumstance, the discussion of this point should be described, and this study should be compared with other related work.
   Growth profile presented and compared

3. Figure 3 shows that several yeast strains still produced high levels of ethanol concentration at 120 g/L sugar. What will happen if the concentration of sugar is higher than 120 g/L? Sugar concentrations at approximately 200 to 220 g/L are applied in industrial ethanol production. Therefore, further experiments on the sugar concentrations in ethanol production should be performed.
   Included

4. The authors compared the results of this study with those of references [50] and [26]. Based on the information in references [50] and [26], these two references used different concentrations of sugars (not 40 g/L) and also different temperatures (not 30 °C). The ethanol produced in these two references is also higher than that mentioned

in the manuscript. Therefore, please clarify this point and clearly describe the correct information in the manuscript.

Clarified

5. Section "Variable effects on bioethanol yield of isolate 9Li2", the information in this section is not correlated with the results in Figure 7. For instance, Figure 7A is the interaction of temperature and pH, not temperature and time, or Figure 7B is the interaction of time and pH, not time and temperature, as mentioned in the manuscript. Please recheck and correct.
   Corrected

6. Further study using lignocellulosic materials should also be performed.
   - We plan to use this promising yeast in conjunction with pentose fermenter yeast for additional research in the future.
   - We have included it as a recommendation for future work.

Conclusion:

1. The authors mentioned in the last sentence that "The simultaneous saccharification and fermentation of lignocellulose materials requires the use of microorganisms able to function at high temperatures, along with pentose fermenter yeasts, and this study strongly suggests utilizing these yeasts". This sentence is overclaimed since the potentially isolated yeast, *S. cerevisiae* 9Li2, can not utilize xylose and also has less tolerance to acetic acid. Furthermore, how much this isolate produces ethanol at high temperatures has yet to be tested. Please reconsider and highlight the novelty of this study.

- S. cerevisiae 9Li2, the selected yeast, is unable to use xylose; however, the isolate demonstrates a high level of acetic acid tolerance, withstanding up to 1 percent. The acetic acid tolerance of the prior data is only reported at 0.3 percent in Table 2 without showing a growth profile. The other isolates in the present study were unable to demonstrate any growth above 0.3 percent. In this, we are recommending using this isolate with xylose fermenter yeasts to produce ethanol.
- With desirability of 0.819, S. cerevisiae 9Li2 produced a significant amount of ethanol (14.8768 g/L ~ 15 g/L) at the optimal temperature (40 °C), pH (5.167), and duration (72 hours).
- Novelty: To the best of our knowledge, no *S. cerevisiae* has been isolated from the litter that was taken from natural forests that did exhibit growth (at 60%) or ethanol synthesis at high temperatures (40 °C) or produce ethanol at sugar concentrations 220 g/L.
- Being among the first research to concentrate on the isolation of wild Yeasts from samples for production of ethanol in southwest Ethiopia makes this work significant.

2. Suggestions for further studies regarding the study's findings should be mentioned in the manuscript.

Further investigation will be necessary to understanding the genomic contents and potential utilization of the selected yeast isolate's on lignocellulosic materials as well as their ability to withstand harsh conditions.

Thank you for submitting your reviewer report===International Journal of Energy Research

Astrilia Damayanti <astrilia.damayanti@mail.unnes.ac.id>

7086047: Thank you for submitting your reviewer report
1 message

Sharon Habacon <sharon.habacon@hindawi.com> Fri, Feb 23, 2024 at 6:07 AM
To: astrilia.damayanti@mail.unnes.ac.id

WILEY

Dear Dr. Damayanti,

Thank you for submitting your reviewer report on 7086047 titled "Optimization of Bio-ethanol Production Using Response Surface Methodology for Stress-Tolerant Wild Yeasts Isolated from Natural Forests" by Anbessa Dabassa Koricha, and for taking the time and effort to review this manuscript for International Journal of Energy Research.

Kind regards,
Sharon Habacon
International Journal of Energy Research

Optimization of Bio-ethanol Production Using Response Surface Methodology for Stress-Tolerant Wild Yeasts Isolated from Natural Forests

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Abstract

Yeasts are important microbes for ethanol production, but their effectiveness varies depending on the physicochemical parameters of the fermenting matrix. Therefore, the current study assessed ethanol production using stress-tolerant yeasts isolated from natural forests of Southwest Ethiopia. Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to identify the isolates. Design-Expert 11.1(Response Surface Methodology) was used to optimize the fermentation condition for effective ethanol production. A total of 406 yeasts were isolated, of which 67% were capable of ethanol production while 33% were no producers. The strongly fermentative isolates belonged to three genera: *Saccharomyces cerevisiae, Meyerozyma guilleirmondii, and Candida pelliculosa*. *S. cerevisiae* 9Li2 and 35L1 were excellent candidates for ethanol production, with *S. cerevisiae* 9Li2 producing the highest ethanol (19.05 g/L) at optimal conditions (pH 5.09, 61.19 hours, and 30°C), while *S. cerevisiae* 35L1 produced maximum ethanol (19.03 g/L) at pH 4.92, 30.03 °C, and 71.55 hours. On the other hand, *S. cerevisiae* 9Li2 was able to ferment 40 g/L of glucose to produce 15 g/L of ethanol at 40 ^{o}C with 73% efficiency. This work shows that multi-stress-tolerant *S. cerevisiae* isolates can thrive in harsh environments and should be enhanced for industrial bioethanol production exploiting their promising fermentation capacity.

Keywords: Ethanol, Fermentation, Optimization, *Saccharomyces cerevisiae*, stress tolerant yeast, wild yeast

Highlights

- Ethiopian forests are potential sources of novel wild yeasts for the production of ethanol.
- By incorporating environmental parameters and stress-tolerant wild yeasts through RSM, ethanol production efficiency is increased.
- Multi-stress-tolerant isolates of *S. cerevisiae* were found fermenting sugar at 40°C and produced significant amount of ethanol.
- Wild yeasts from natural forests can ferment carbohydrate, produce ethanol under stressful environments.

1. Introduction

The biofuel industry relies heavily on ethanol production that requires efficient and stress tolerant yeast isolates. The use of wild yeasts that can withstand harsh environmental conditions, including high temperatures, low pH levels, oxygen, and incubation time, is crucial to improving ethanol production [1–3]. To achieve this, researchers employ Response Surface Methodology (RSM), a statistical strategy that simultaneously optimizes many variables. Researchers can find out how these variables interact and affect ethanol yield by adjusting variables like temperature, pH, nutritional content and substrate concentration [4,5]. Accordingly, among methods used to increase the effectiveness of ethanol production is to combine RSM with wild yeasts that can withstand stress. Yeasts' distinctive genetic and metabolic characteristics make them capable of producing ethanol from biomass substrates with high efficiency. Thus, researchers using RSM to investigate the complex link between environmental conditions and yeast performance in order to find the most successful yeast isolates for ethanol production [4,5].

Optimizing ethanol production through RSM [6,7] and stress-tolerant wild yeasts supports sustainable bioethanol development and aligns with global efforts to reduce greenhouse gas emissions and fossil fuel dependence. By leveraging advanced statistical techniques like RSM [1,7,8] and stress-tolerant wild yeasts, one can pave the way for a greener and more efficient bioenergy industry

Further exploration is required to fully harness the potential of wild yeast in ethanol production and determine their stress tolerance. Research efforts are focused on understanding their genetic diversity, metabolic pathways, and fermentation characteristics. Scientists are trying to create specialized fermentation techniques that maximize ethanol output, boost product quality, and

increase overall sustainability in the biofuel business by discovering the secrets of these wild microbes.

We describe the discovery and physiological characterization of a thermo-tolerant *S. cerevisiae* isolate that can grow at 40 ℃ under the aforementioned circumstances and produce significant quantities of ethanol. Our goal was to fill some of the research gaps that exist in this area, such as the lack of information on stress-tolerant wild yeast isolates, the use of Response Surface Methodology (RSM) to fine-tune process parameters that affect stress tolerance, and the blending of various pretreatment techniques and fermentation conditions to improve results.

In general, integrating the Response Surface Methodology with stress-tolerant wild yeasts is a promising approach to maximize ethanol production under optimum conditions. Researchers can now fully comprehend the intricate relationships between environmental factors and yeast function thanks to this groundbreaking methodology. By doing this, we can fully realize the benefits of renewable and environmentally friendly biofuels, paving the way for a more sustainable future.

2. Materials and Methods

2.1. Study area description and Sample Collection

The study sites were three natural forests in Jimma and Iluababor zones, Southwest Ethiopia [Fig. 1]. Belete-Gera Forest, located at 70 30'–70 45' N and 36^0 15'–36^0 45' E, has an annual rainfall of 1800–2300 mm, mostly from June to September. The temperature varies from 15 to 22°C throughout the year [9]. Boter Becho Forest lies between 80 12'–80 37' N and 37^0 06'–37^0 29' E with an altitudes ranging from 1500 to 3100 m above sea level and. The yearly precipitation in the area is 120–2000 mm [10]. Yayu Forest, situated at 8°21'–8°26' N and 35°45'–36°3' E, is found within an altitude of 1200–2000 m [11].

Bark and leave samples were collected from surfaces of different plant species. Bark samples of old logs of trees were collected from 0 to 3 m at different gradients of the stem base of each log. Whereas the rhizosphere soil was collected at a depth of 0–10 cm from underneath the selected old logs after leaf litter samples were collected. Overall, samples of bark (n=158), soil (n=121),

litter (n=66) and leaves (n=61) were collected between September 2021 and June 2022. All samples were transported to research laboratory using ice boxes, and stored at 4°C until used for different analysis.

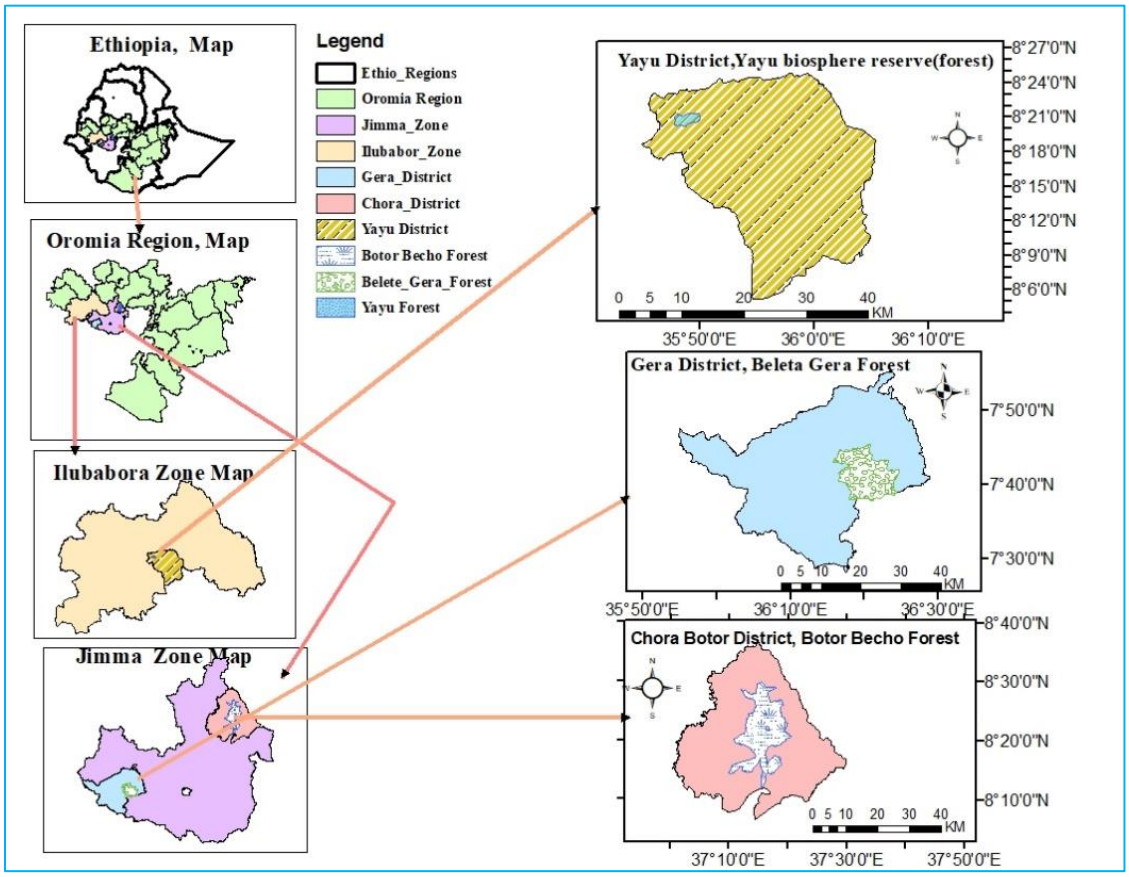


Fig. 1. Map of the study area[9,10,11]

2.2. Isolation of wild yeasts.

Four grams of solid materials were cut into smaller pieces before added to the enrichment medium containing 1% yeast extract, 2% peptone, and 2% D-glucose, along with 1M HCL in 45 ml capacity flask. Each sample was cultured for 7 to 14 days at 30 °C until signs for the beginning of fermentation were noticed. Then, about 100 µl of actively fermenting cultures were diluted and dispensed on YPD agar containing chloramphenicol (100 mg/L) followed by incubation at 30 °C for 2 to 3 days [12]. The colonies were further purified by repeated streaking on YPD agar medium. Morphologically distinct colonies were randomly picked and characterized [13]. Microscopy was used to examine the isolates' cell morphology (shape and

size); and any non-yeast isolates (such as bacteria) were excluded from further analysis [14]. The pure isolates were stored at -70°C in micro-tubes (1.8 mL) half-filled with YPD broth that was fortified with glycerol (50%, w/v) for further analysis [15].

2.3. Characteristics of fermentation using different carbon sources

The ability of yeast isolates to ferment glucose, galactose, fructose, maltose, lactose, xylose, and sucrose was evaluated following standard method as suggested by Zaid et al. [16] using the Wikerham medium: containing peptone (10 g/L), yeast extract (5 g/L), phenol red (24 mg), distilled water (1 L). The sugars were dissolved at 2% (w/v) concentration. About 0.1 mL of active yeast cells from each isolate were separately inoculated in to test media pre-prepared in Durham tubes and incubated at 30 °C for 4 days without any agitation.

2.4. Selection of wild yeasts under stress conditions

The isolates' ethanol tolerance was tested by inoculating each yeast isolate ($1x10^7$cells/ml) into YPD broth supplemented with varied concentrations of absolute ethanol (v/v) (5, 10, 14, 16, and 18%) according to Rahman et al. [4]; and incubating the culture at 30°C for 24–72 hours at 150 rpm. The yeast cultures were then streaked onto YPD agar and allowed to grow for 72 hours at 30°C. Based on the level of cell growth at 72 hours, the yeast isolates were categorized into three groups (highly tolerant, moderately tolerant, and mildly tolerant) following the method suggested earlier [17]. For further testing, yeast isolates with a high ethanol tolerance ranging between 12 and 18 percent (v/v) were chosen.

The temperature tolerance of ethanol-tolerant yeasts was evaluated by spreading the yeast isolates separately on YPD agar and incubating them at 37, 40, 42, 44, and 45°C for 72 hours [18,19]. Through direct observation for the formation of colonies on YPD agar plates, the presence or absence of growth of the yeast isolates was verified. For additional tests, those yeast isolates that tolerated temperatures of at least 40°C were considered.

The yeast isolates' osmo-tolerance and pH tolerance were quantified by transferring portions of actively growing yeast cultures ($1x10^7$cells/ml) into YPD broth that had been adjusted to various pH values (i.e., 2, 3, 4, 5, 6) and glucose concentrations (i.e., 30, 40, 50, and 60%) [13]. They were then incubated at 30 °C for 72 hours at 150 rpm. Finally, fermentative yeasts that thrived at

conditions of 14–16% (v/v) ethanol concentration, 40°C, pH 2.0, and 60% glucose concentration were selected.

2.5. Sugar utilization during ethanol fermentation

One hundred mL (100 mL) of YPD broth containing 4% D-Glucose and $1x10^7$cells/ml yeast cells were inoculated, and the mixture was then incubated at 30 ^oC for 72 hours. The isolates utilization of the sugar was tested at 5, 10, 15, 20, 24, 48, and 72 hours of fermentation [8].

2.6. Effect of glucose concentrations on ethanol production

This test was conducted following the methods outlined by Costa et al. [20] and Vázquez et al. [21]. Accordingly, different glucose concentrations (20, 40, 60, 80, 100 and 120 g/L) were used with YPD broth containing 1% yeast extract and 2% peptone to which approximately $1x10^7$cells/ml yeasts were inoculated and incubated at 30 ^oC to fermentations for three days.

2.7. Yeast Identifications

Based on the distinctive protein profiles for each microorganism, Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has been used as a quick and accurate method for the selected yeast identification. The identification was done according to the technique suggested by Ferreira et al [22].Briefly, a single colony was pipette-transferred from the plate to a 1.5-ml tube (Eppendorf, Germany) and well mixed with 300 ml of water. The mixture was then centrifuged at 15,500 g for 2 minutes with the addition of 900 ml of 100% ethanol, and the supernatant was discarded. The pellet was air-dried for an hour at room temperature. The pellet was then thoroughly mixed with 50 ml of formic acid (70% v/v) before being added to 50 ml of acetonitrile. The mixture was centrifuged once more at 15,500 g for 2 minutes. An area of the steel target was covered with one microliter of the supernatant, which was then air-dried at room temperature. According to Xiong et al. [23], each sample was covered in 1 microliter of the matrix solution (cyano-4-hydroxycinnamic acid) and allowed to air dry. Then, the measurements were performed on an EXS300 MALDI/TOF mass spectrometer. Using modified score values suggested by the manufacturer, the identifications were categorized as follows: A score value >2 indicated species identification; a score value between 1.7 and 1.9 indicated genus identification; and a score value of 1.7 indicated no identification.

2.8. Batch fermentation, Experimental design and statistical analysis

The YPD broth (yeast extract 1%, peptone 2%, and D-glucose 4% w/v) was used in batch fermentation for 48 hours to examine the effects of temperature (30, 35, and 40 ^{0}C) and pH (4, 5, and 6) on ethanol output [24]. Accordingly, yeast culture ($1x10^7$cells/ml), were inoculated into YPD broth containing 4% dextrose and samples were drawn at 24 hours interval (24, 48, and 72 hours) in the course of fermentation for determination of levels of ethanol produced.

The Central Composite Design (CCD) was applied using Design Expert 11.1.2 (Stat Ease, Inc., Minneapolis, USA) in the response surface methodology tests, which involved growing yeasts in 250 mL Erlenmeyer flasks using 100 mL YPD. The total number of experiments generated by CCD from 3 factors at 3 levels with six replications at the center point to evaluate the pure error was determined by the simple formula [20 = 2k + 2k + 6], where k is the number of independent variables (k = 3). After the experiments were conducted, a response surface experiment was conducted to generate a prediction model to detect interactions among the design factors (independent variables) and optimize the process to identify the optimum independent variables with the maximum yield of bioethanol [7,8]. The quadratic polynomial denoted by the equation given below was the model employed in this investigation to estimate the response surface:

$$Y = \beta_0 + \beta_1X_2 + \beta_2X_2 + \beta_3X_3 + \beta_1\beta_2X_1X_2 + \beta_1\beta_3X_1X_3 + \beta_2\beta_3 X_2X_3 + \beta_{11}X_1^2 + \beta_{22} X^2_2 + \beta_{33} X_3^2 + \varepsilon$$

……..……**Equation 1**

Where, Y = g/L of ethanol produced (dependent response), β0 = intercept (the value of the fixed response at the center point of the design), β1, β2, and β3= Temperature, pH, and time for linear, quadratic, and interaction regression coefficients, respectively. X_1, X_2, and X_3 = independent variable (factors) for temperature (degree centigrade), time (hours), and pH, respectively; ε = random experimental error expected to have a zero mean.

Based on findings of the preliminary study, the levels were chosen. The design factors (variables) X_1 [30 and 40 ^{o}C], X_2 [24 and 72 hr], and X_3 [4 and 6] are those with low values of -1 and high levels of +1. The center values; zero level determined for the experimental design were 35°C, 48 hours, and 5, respectively, for X_1, X_2, and X_3.

2.9 Ethanol estimation

After isolates revealed successful fermentation using Durham tubes, they were further tested for fermentation in the YPD culture medium as suggested in earlier work [16]. A digital refractometer (Bellingham + Stanley RFM300-M) was used to measure the utilized sugar having drawn samples at 0, 24, 48, and 72 hours [25–27]. Brix was measured before the experiment began and after it was finished. Based on the recommended method, published by the Organization International of Vine and Wine (Method OIV-MA-AS312-01A), the percentage alcohol (ABV% v/v) of the resulting product was calculated. The fermented products were centrifuged at 10,000 g for 5 minutes in order to extract the ethanol, and the supernatant was also qualitatively tested for ethanol using the potassium dichromatic method [25,28]. Finally, ethanol concentration was calculated by comparing optical density readings made at 600 nm using a UV-Vis spectrophotometer (Analytik Jena, Germany) with a built-in calibration curve using various concentrations (1–10% v/v) of absolute ethanol as reference.

2.10 The measurement of ethanol concentration, ethanol yield, volumetric productivity, and fermentation efficiency

Ethanol concentration, ethanol output, productivity, and efficiency was determined using YPD with 40g/L of glucose according to Tesfaw et al [8]. For ethanol measurement (g/L), samples were taken at 5, 10, 15, 20, 24, 48, and 72 hours. The ratio of sugar utilized (g/L) to ethanol concentration (g/L) was used to define ethanol yield (g/g). The maximum ethanol concentrations (g/L) at the appropriate fermentation time (h) were used to determine ethanol productivity. The theoretical maximum ethanol yield was found to be 0.51 g of ethanol per gram of glucose. The ratio between the ethanol yields (g/g) was used to calculate the sugar conversion efficiency (%). The percentage of the original sugar concentration that was consumed in sugar (%) was calculated following standard methods described earlier [8,29] .

3. Results and Discussion

3.1. Yeast isolation and screening

3.1.1. Yeast isolation

A total of 406 wild yeasts were isolated from natural forest substrates, including tree barks, rhizosphere soil, leaf litters, and actively growing leaves. The highest number of yeast colonies was recorded from tree barks (n=158) followed by rhizosphere soils (121), litters (66), and leaves (61) (Table 1). According to earlier research reports, yeast species with exceptional ethanol fermentation capabilities have been isolated from leaves, flowers, sweet fruits [30], tree exudates, grains, roots, insects, and locally fermented foods and drinks [13,31,32]. This is accounted to their interaction with sugar-rich substrates and gradual adaptation to the environments [13].

3.1.2. Screening of ethanol positive yeasts in glucose

In the confirmatory test for production of ethanol, 271 (67%) isolates were able to produce gas from glucose. The majority, 114(28%), of glucose-fermenting yeasts were isolated from tree barks followed by isolates from rhizosphere soil 84(21%), and the least number37 (9%, each) was isolated from leaf and litter (Table 1).

Table 1: Sample source, number of yeasts isolated and their efficiency of glucose fermentation to ethanol after 72 hours of incubation at 30^{o}C

Sample source	Number of samples (%)	Number of yeast isolates (%)	Number of gas producers (%)
Bark	67(33.5%)	158 (39%)	114 (28%)
Leaf	32 (16%)	61 (15%)	36 (9%)
Litter	52 (26%)	66 (16%)	37 (9%)
Rhizosphere soil	49 (24.5%)	121 (30%)	84 (21%)
Total	200 (100%)	406 (100%)	271 (67%)

3.1.3. Screening of stress tolerant yeasts

The growth characteristics and carbohydrate fermentation profile of wild yeasts are as given below (Table 2). Furthermore, among thirty six ethanogenic wild yeast isolates examined for their resistance to various stressful conditions, more than half were found resistant to pH 2

(n=17), 60% osmotic pressure (n=34) and very few to 0.3% acetic acid (n=6). In addition, a total of 26 isolates exhibited resistance to ethanol doses ranging from 12% to 18%.

Table 2: Growth and fermentation profile of potential ethanol producer wild and baker's yeasts against different stress factors

Factors		Ethanol production among wild yeast isolates and bakery yeasts				
		Wild yeast				Bakery yeast
		49B1	46Li1	9Li2	35L1	
High Ethanol tolerance	14%	+	+	+	+	+
	16%	+	+	+	+	-
	18%	+	+	-	-	-
High Temp. tolerance	40^{o}C	+	+	+	+	+
	42^{o}C	+	+	+	+	-
	45 ^{o}C	-	+	+	-	-
Osmotic tolerance (D-glucose)	50%	+	+	+	+	+
	60%	+	+	+	+	+
Acetic acid tolerance	0.2%	+	+	+	+	+
	0.3%	-	+	+	+	-
pH tolerance	pH,2	+	+	+	+	+
	pH,3	+	+	+	+	+
Carbohydrate fermentation	Glucose	+	+	+	+	+
	Galactose	+	+	+	+	+
	Fructose	+	+	+	+	+
	Sucrose	+	+	+	+	+
	Maltose	-	+	+	+	+
	Lactose	-	-	-	-	-
	Xylose	-	-	-	-	-

Where, "+"= growth; "-"= no growth

Besides, 22 of the 36 yeast isolates tolerated temperature as high as 42 and 45 ^{o}C (Fig. 2). Four isolates, including *Saccharomyces cerevisiae* 9Li2 and 35L1, *Meyerozyma guilliermondii* 49B1, and *Candida pelliculosa* 46Li1 that displayed the highest stress tolerance in all parameters were chosen for further characterization and ethanol production capability as described earlier. Based on the efficiency they displayed, two isolates (*Saccharomyces cerevisiae* 9Li2 and 35L1) were selected for optimization of the fermentation condition based on combination of the

aforementioned parameters, time it took to produce ethanol and the rate at which the substrate was converted to ethanol.

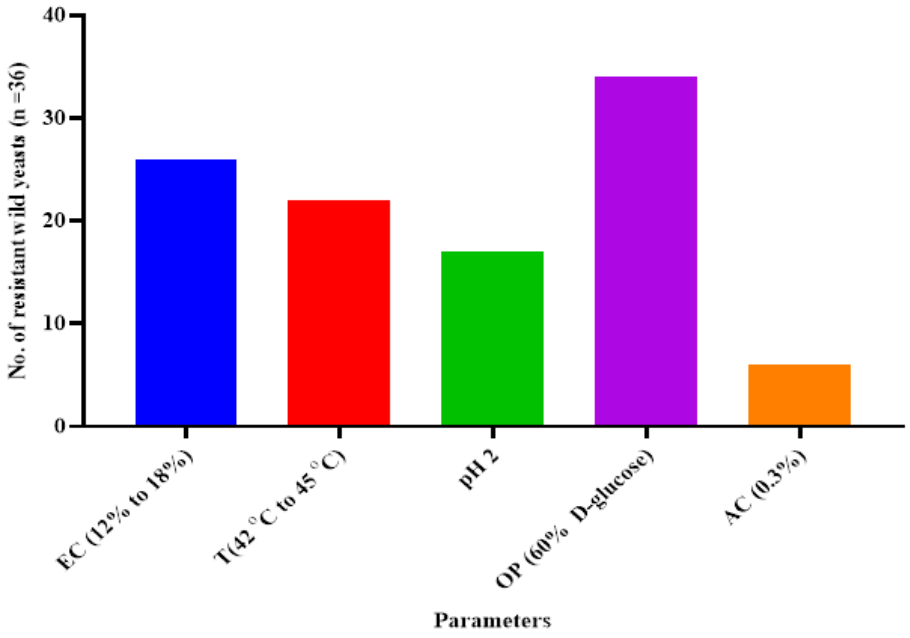


Fig.2 Stress tolerance of fermentative wild yeasts isolated from natural forests under different stressful conditions

Where EC=Ethanol concentration, T= Temperature, OP= Osmotic Pressure, AC= Acetic acid

Saccharomyces cerevisiae 9Li2 and 35L1 were moderately tolerant to 16% (v/v) ethanol. These results agreed with those of Hawaz et al. [19] who reported yeast isolates tolerance to ethanol concentrations as high as 16% (v/v). The finding of this study is also consistent with previous studies on thermotolerant yeasts isolated from some sources (soil, fruits, and flowers) in Vietnam [33]. In contrast to this study, Rahman et al. [34] reported relatively lower tolerance (12%) to ethanol of *Saccharomyces* species. High ethanol concentrations are responsible for inducing cellular stress in yeast metabolism and reducing cell growth rate and viability [35], consequently leading to low productivity. The inability of *S. cerevisiae* to grow in media high in alcohol content results in drop in ethanol production [35]. However, industrial applications require yeasts that can withstand stressors and remain viable throughout the fermentation process [36,37].

In the current study, two of the newly selected *S. cerevisiae*, namely *S. cerevisiae* 9Li2 and 35L1 were able to grow well at pH 2.0. During fermentation process for the production of ethanol,

acidophilic yeasts help avoid bacterial contamination [13,38]. As shown in Table 2, the selected yeasts (isolate 9Li2 and 35L1) were resistant to high-temperature scale of 45 and 42 °C, respectively. This result is consistent with study by Nasir et al. [39] who reported that yeasts isolated from fruit samples were thermo-tolerant and could survive temperature as high as 44 °C. It is also consistent with the findings of Costa et al.[20] and Kumar et al.[1], who showed that yeast isolates can withstand temperatures of 45 °C. Thermo-tolerant yeast isolates have also been reported from Vietnam and Ethiopia [17,26,40]. Because they have a lower risk of bacterial contamination and do not require the full costs associated with thermal maintenance, distillation, and the addition of the exogenous enzyme, the use of thermo-tolerant yeasts are advantageous for ethanol production [41–43].

Among yeast isolates characterized in the current study, *S. cerevisiae* 9Li2, *S. cerevisiae* 35L1, and *Candida pelliculosa* 46Li1 thrived at 0.3% acetic acid unlike the baker's yeast and *M. guilliermondii* 49B1 yeast that grew well at 0.2% acetic acid (Table 2). As reported by *Narendranath* [44], *S. cerevisiae* can grow more slowly in settings with acetic acid concentrations of at least 0.6% w/v as it dissociate and enter cells in its protonated state, ultimately reducing the intracellular pH. Acetic acid is a well-known antibacterial chemical present in lignocellulose hydrolysates [45].

The examination into the osmo-tolerance of the yeast isolates was carried out at various glucose concentrations ranging from 30 to 60% (w/v) (Table 2). Sixty percent (60%) (v/v) sugar content was well tolerated by 34 yeast isolates out of the total 36 isolates. The two chosen yeast isolates could also thrive well at 60% glucose content. For industrial production of ethanol, yeasts that tolerate high-sugar environments are advantageous in terms of cost and yield. The yeast's capacity to endure osmotic stress and high ethanol concentrations is essential for successful fermentation [46,47].

Nearly all of the chosen isolates were able to grow and ferment glucose, galactose, fructose, and sucrose vigorously, but none of them were able to use xylose or lactose (Table 2). Unsolvable problems exist in the manufacture of bioethanol because industrial yeast cannot ferment pentose carbohydrates [13] with different yeast isolates having a variety of sugar usage profiles.

3.2. Yeast identification

The identification of yeasts was done using MALDI-TOF MS, EXS300. The stress-tolerant yeast isolates were categorized under three genera. These include two isolates belonging the genus Saccharomycese, including *S. cerevisiae* 35L1 isolated from leaf of Baddeessaa (*Syzygium guineensa*) in the Boter-Becho forest and *S. cerevisia* 9Li2 isolated from trash near Waddeessaa (*Cordia africana*) in the Beleta-Gera forest. The remaining were identified as *Candida pelliculosa* 46Li1 isolated from litters around Baalantaa'ii (*Celtis Africana*) of Yayu forest, and *M. guilliermondii* 49B1 isolated from the bark of Lookoo (*Diospyros abyssinica*), located in Yayu forest (Table 3).

Table 3: Yeast isolates capable of producing ethanol under 40g/L glucose concentration incubated at 30^{o}C for 72hr

Yeast isolates	Sample Sources	Isolates Identity	Study Sites	MALDI-TOF MS Scores
35L1	Leaf	*Saccharomyces cerevisiae*	Boter-Becho	2.22
46Li1	Leaf litter	*Candida pelliculosa*	Yayu forest	2.02
9Li2	Leaf litter	*Saccharomyces cerevisiae*	Belete-gera	2.11
49B1	Tree bark	*Meyerozyma guilliermondii*	Yayu forest	1.87

3.3. Effect of glucose concentration on bioethanol production

To determine whether yeast isolates had the potential to produce ethanol at very high gravity, the impact of glucose content on those isolates was examined. Maximum ethanol production was achieved by *S. cerevisiae* 35L1 and 9Li2 at a glucose concentration of 120 g/L at 30 °C (Fig.3). With other yeast isolates and baker's yeast, however, the ethanol concentration dropped as the glucose concentration increased. In the ethanol fermentation process, *S. cerevisiae* (control) was less osmo-tolerant than *S. cerevisiae* 35L1 and 9Li2, which may lead to low bioethanol production. A rise in sugar concentration leads to an increase in cell density up to a certain threshold, but after that point, the number of cells decreases [13]. As observed both in this work and others reported elsewhere [48], in *S. cerevisiae's* 9Li2 rapid glucose uptake was directly correlated with ethanol yield. For the two yeasts (35L1 and 9Li2), the pattern of increment in

ethanol concentration with increment in sugar concentration was the same; however, the pattern of lowering for other yeasts after the maximum ethanol production was different (Fig. 3).

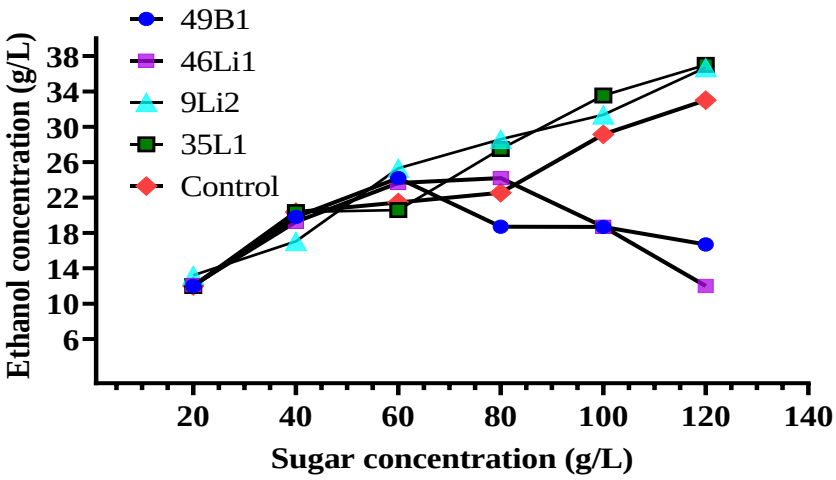
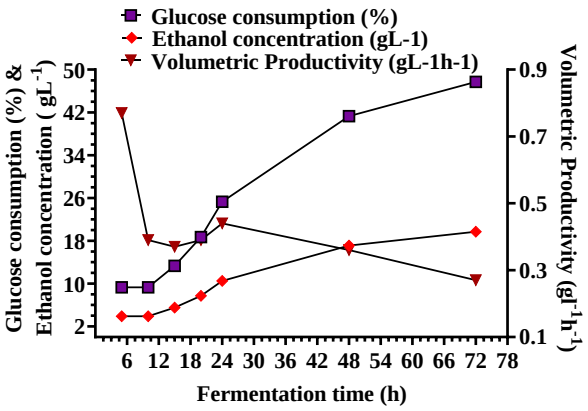


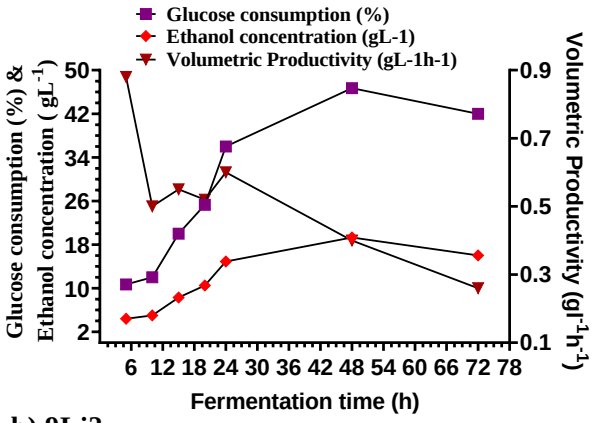
Fig. 3. Effect of glucose concentration on ethanol concentration in three-day incubation at 30°C

3.4. Ethanol Concentration, yield of ethanol, volumetric productivity and fermentation efficiency of selected yeasts

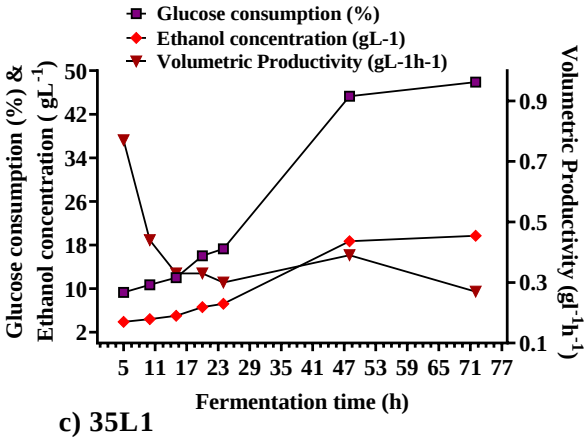
Selected yeasts were grown at 30°C for 5 to 72 hours while their glucose consumption, ethanol content, and volumetric productivity were assessed (Figure 4a - e). After 72 hours, isolates 49B1 and 35L1 produced the highest levels of ethanol. The fast conversion of carbohydrates to ethanol by isolate 9Li2 may provide a competitive advantage. This is consistent with Nadir et al. [49], where the highest ethanol concentration was reached after 64 hours and then decreased by 10% after 72 hours. Considering the glucose consumption rate, the most rapid glucose consumption was observed in the isolate 9Li2 within 24 hours suggesting that under stress conditions, the microbes require more energy sources to efficiently carry out their metabolic activities [37]. Although volumetric productivity exhibited a long-term positive association with isolate 9Li2 and Baker's yeast (Fig. 4b and e, respectively), consumption of glucose and ethanol was considerably influenced by the length of the fermentation process.



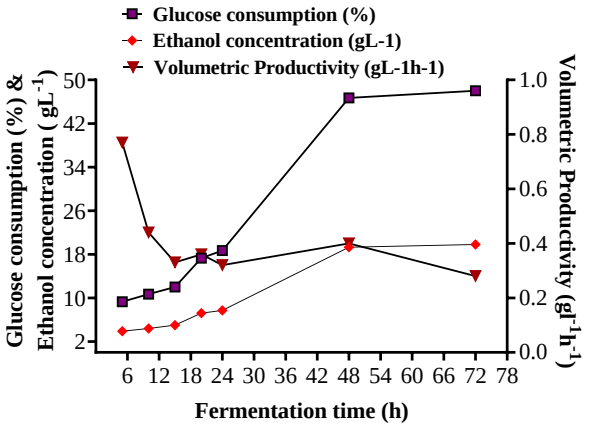
a) 46Li1



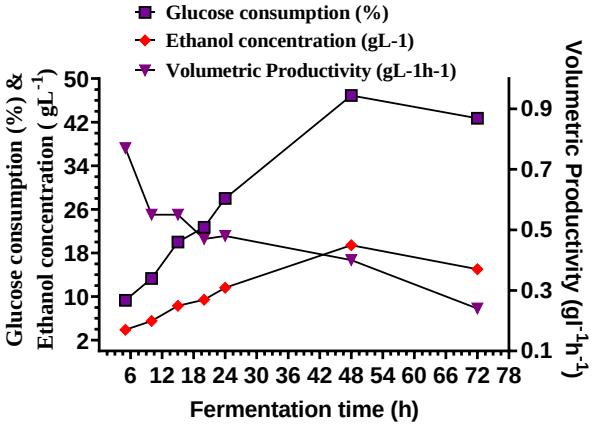
b) 9Li2



c) 35L1



d) 49B1



e) Control (Baker's yeast)

Fig. 4 Efficiency of fermentation for selected yeast isolates: 49B1(a), 46Li1(b), 9Li2 (c), 35L1(d)) and control yeast (e) grown in medium containing 4% glucose at various fermentation times (5–72 hr.) incubated at 30 ^{o}C. The left Yaxes indicate Glucose and Ethanol Concentration while Volumetric Productivity (VP) are plotted at the side of the right Y axis.

The wild yeasts *S. cerevisiae* 35L1 and 9Li2 performed the best in terms of ethanol efficiency, producing ethanol concentrations of 20 and 18.2 g/L from 40 g/L glucose after 72 fermentation hours at 30 ^{o}C, respectively. As compared to previous reports, all isolates in the current study are highly efficient bioethanol producers with better result than those of *S. cerevisiae* DBKKUY-53 (14.5 g/L) [50] and the 4.5 to 13.5 g/L ethanol from 40 g/L glucose reported from 26 yeast isolates by Techaparin et al. [26]. A similar yield was reported by Tesfaw et al.[8] from *S. cerevisiae* ETP53 that produced 20.93 g/L at 30.1 °C incubated for 58.97 hr from the same amount of glucose used in our study. The maximum ethanol yield reported by earlier studies with pure glucose was 0.27 g/g [37] and was significantly lower than the 0.48–0.50 g/g yields obtained in the current study.

3.5. Statistical Optimization for Ethanol Production by selected yeast isolates Using RSM

One of the scientific methods helpful for the development, enhancement, and optimization of industrial processes is the response surface method. It's main goal is to select the optimal operational parameters while keeping within the parameters set out by the operational needs [25]. Accordingly, the effects of temperature, fermentation time and pH were studied as three independent variables affecting bioethanol production in a total of 20 experiments through a centralized composite design. The predicted model is verified experimentally using the actual values of bioethanol production and the corresponding predicted values were presented in Figure 5A for four newly isolated isolates.

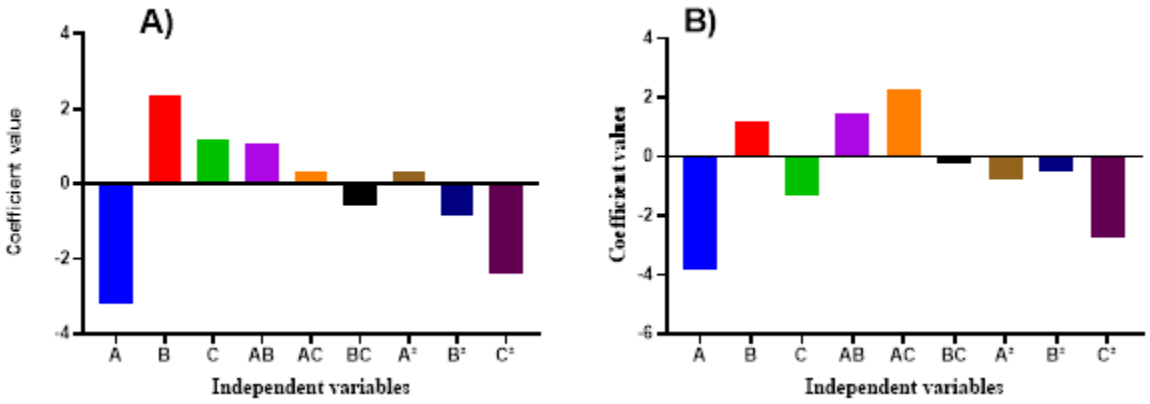


Fig. 5 Pareto graphic illustrating the effect of different variables on the response of bioethanol yield produced by isolate 9Li2 (A) and 35L1 (B) in linear, interactive, and quadratic terms

Pareto charts, particularly useful in experiment design, were used to depict the main and interaction effects of all parameters on the response variable for bioethanol concentration. As shown in Figures 5A and B, temperature had the greatest linearly negative impact on the yield of bioethanol. As the model revealed, after the incubation period indicated in Fig. 5A, the linear influence of pH had the second-largest favorable impact. However, in Figure 5B, the pH's positive linear impact on the bioethanol concentration (g/L) was the greatest. pH had a beneficial impact on the linear effect when 9Li2 was used but had a negative impact on the product when 35L1 was used. Similar to how pH and temperature interacted, the relationship between temperature and fermentation time also showed some positive effects. The strongest negative quadratic effect on the production of bioethanol was caused by pH (Fig. 5A and B).

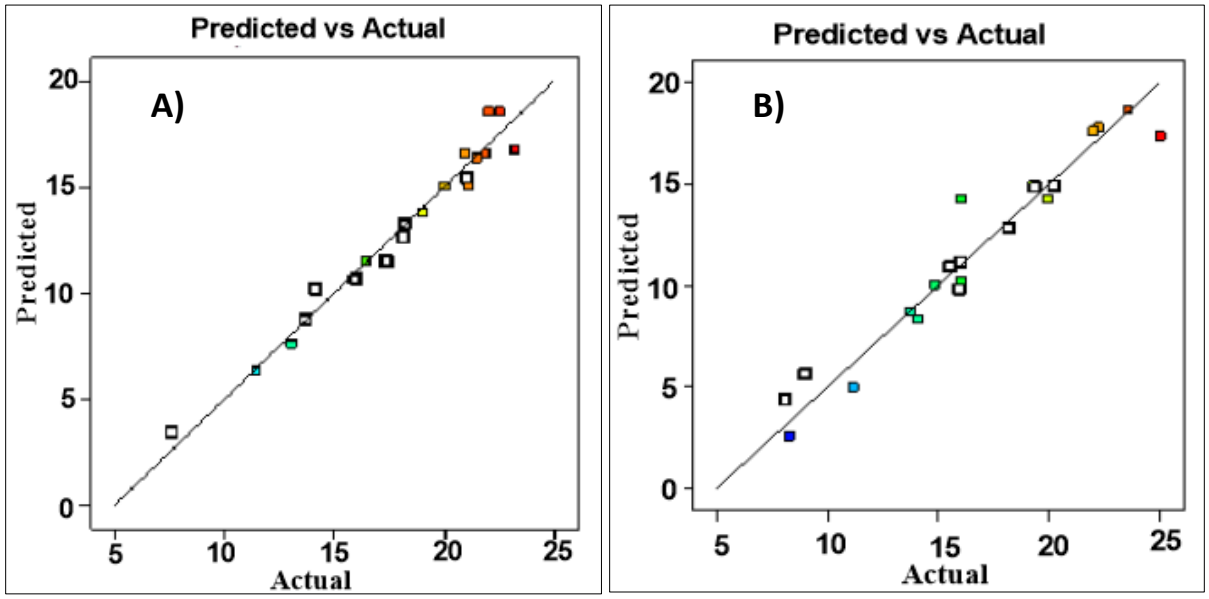


Fig. 6 Actual vs. predicted values of bioethanol yield by isolate 9Li2 (A) and 35L1 (B)

The full design matrix with experimental and expected values of the concentration of produced bioethanol (g/L) is as shown in Fig. 6. The model's efficacy is demonstrated by the high correlation coefficients (R^2), which account for 0.9683 and 0.9427, respectively, between projected and experimental bioethanol production levels by isolate 9Li2 (Fig. 6A) and 35l1 (Fig. 6B). This suggests that the quadratic model, which was employed to describe the mathematical relationship between the independent variables and the dependent response, adequately fits the experimental data. This further implies that the response estimate for the system within the investigated experimental range is highly accurate.

3.5.1. *Saccharomyces cerevisiae* 9Li2

The actual yield and predicted value of the model are presented in Fig. 6A, which illustrates the correlation between bioethanol production by *S. cerevisiae* 9Li2 using expected and experimental values. The quadratic model, as displayed in Table 4 ($P < 0.0001$), was fitted to the data. The level of significance reveals that incubation temperature, duration of fermentation, and pH had significantly high effects on bioethanol production. Moreover, the linear and squared terms for time and pH demonstrated their substantial influence on bioethanol production. Conversely, interactions between time and pH, as well as temperature and time, showed no noticeable effects ($P > 0.05$).

Therefore, by utilizing coded variables for each element, a mathematical model equation for ethanol yields was formulated. This equation not only aids in predicting factors that positively and negatively impact yield but also provides insights into their magnitudes. Equation 2 and Figure 5B reveal that factors B, C, AB, AC, and A^2 positively affect ethanol production, while factors A, BC, B^2, and C^2 have negative effects. The intercepts of this equation (15.0671) allow for precise forecasting of ethanol yield outcomes. Based on these findings, the second-order polynomial equation relating ethanol production (Y) to temperature (A), time (B), and pH (C) can be established.

Ethanol Yield (Y) = $15.0671 - 3.17613A + 2.34852B + 1.15633C + 1.075AB + 0.3275AC - 0.6BC + 0.331828A^2 - 0.850026B^2 - 2.40656C^2$ …… Equation 2

Where Y is the response variable (g/L) and A, B and C are temperature, pH and incubation time, respectively

Table 4 ANOVA and Fit statistics for Quadratic model for *S. cerevisiae* 9Li2

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	330.02	9	36.67	33.96	< 0.0001	Significant
A-Temperature	119.05	1	119.05	110.27	< 0.0001	
B-Time	79.95	1	79.95	74.05	< 0.0001	
C-pH	20.93	1	20.93	19.39	0.0013	
AB	9.25	1	9.25	8.56	0.0151	
AC	0.8580	1	0.8580	0.7948	0.3936	
BC	2.88	1	2.88	2.67	0.1335	
A^2	0.9285	1	0.9285	0.8600	0.3756	
B^2	9.34	1	9.34	8.65	0.0148	
C^2	70.43	1	70.43	65.24	< 0.0001	
Residual	10.80	10	1.08			
Lack of Fit	10.06	8	1.26	3.39	0.2476	not significant
Pure Error	0.7410	2	0.3705			
Cor Total	340.82	19				
Fit statistics						
R^2 = **0.9683,** Adjusted R^2 = **0.9398,** Predicted R^2 = **0.8389,** Adeq Precision = **20.5740**						

df, Degrees of freedom; F, Fisher's variance ratio; Cor Total, Totals corrected for the mean; P, probability value($P < 0.05$; significant at 5% level); Coefficient variation, CV ; Standard Deviation, Std. Dev

Variable effects on bioethanol yield of isolate 9Li2

When examining the response surface and the contour plot in Figure 7A, it is evident that ethanol production decreases as time and temperature increase, indicating a decrease in yeast tolerance to extended exposure to higher temperature. Additionally, bioethanol yield decreases when the pH falls below or exceeds 5. The outcomes shown in Figure 7B show that initially increasing time and temperature lead to an increment in ethanol production, but exceeding 35°C has a negative impact. The data in Figure 7C suggests that the interaction between pH and time does not significantly affect bioethanol production. By considering the interaction of temperature (30 °C), pH value (5.09), and incubation time (61.19), optimal ethanol production of 19.051 g/L can be achieved. These positive results are consistent with earlier research. For instance, Tesfaw et al.[8] used *S. cerevisiae* ETP53 at temperature (30.1 ^{o}C), pH value (5.13), and incubation period (58.97 h) to generate 20.93 g/L ethanol concentration from 40 g/L glucose. In contrast to this study, however, Ali et al. [29] discovered that *S. cerevisiae* (MTCC 170) could produce ethanol

at a maximum concentration of 10.5 g/L under optimal conditions of pH 4.5, 30 ^{o}C, and 48-hour incubation period. *S. cerevisiae* 9Li2, on the other hand, was able to ferment 40 g/L of glucose resulting in 15 g/L of ethanol at 40 ^{o}C.

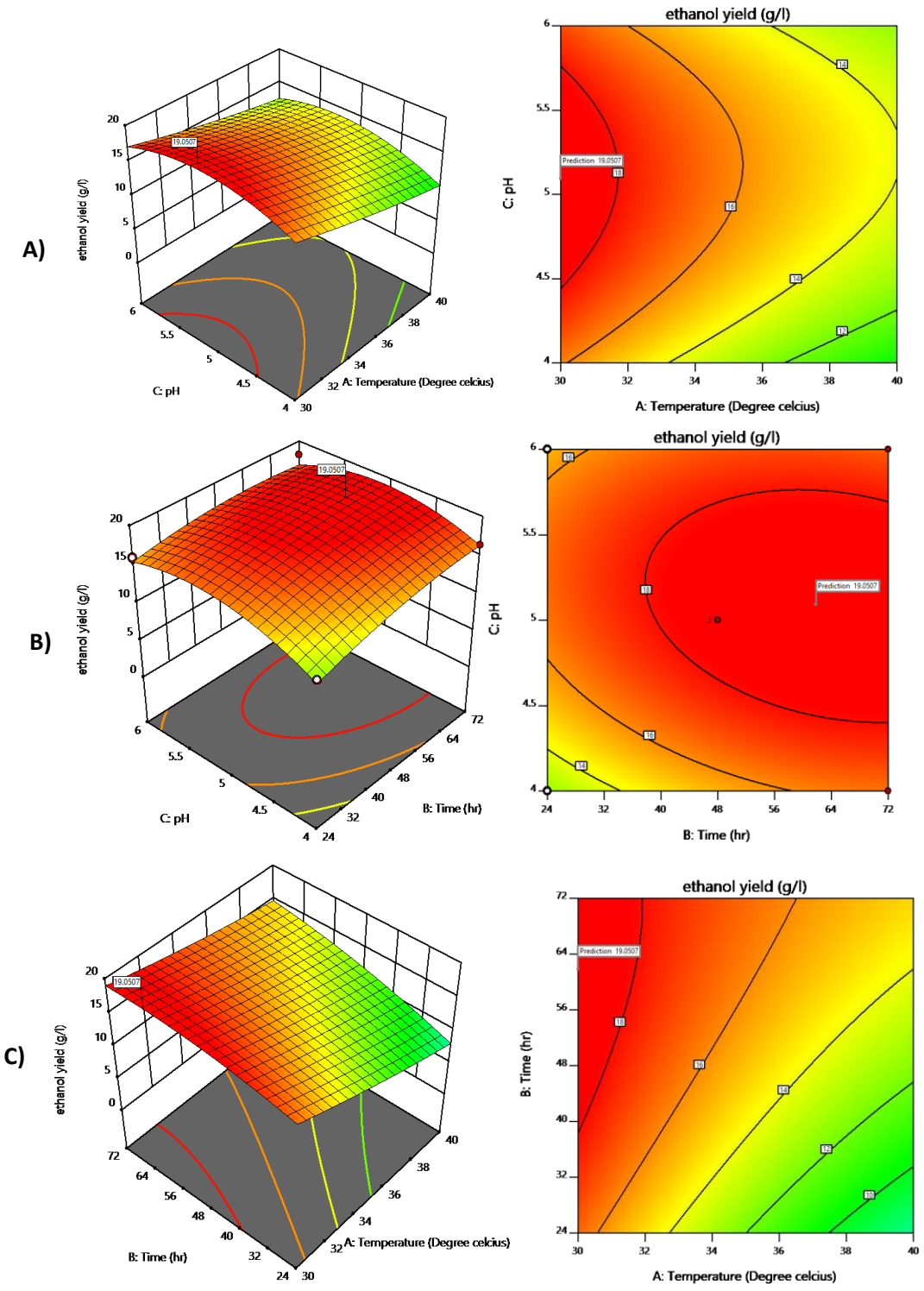


Fig. 7 The response surface (right side) and contour plot (left side) of *S. cerevisiae* 9Li2 to pH vs. temperature (A), temperature vs. time (B) and pH vs. time (C) of ethanol (g/L) produced from 40 g/L dextrose.

3.5.2. *Saccharomyces cerevisiae* 35L1

Fig.6 displays the actual yield and the expected value produced by the model. In addition, ANOVA analysis showed the magnitude of the F-value (18.27) and the low probability value (< 0.0001), proving the significant model fit. The model presented in Table 5 for 35L1 has a high R-squared value of 0.9427, which explains 94.27% of the variation in the response, and a high value of the adjusted coefficient of determination (adjusted R^2 = 0.8911), which shows a correlation between the observed and predicted values, suggesting a high significance of the model (Fig. 6B).

Table 5: ANOVA and Adequacy test for Quadratic model for *Saccharomyces cerevisiae* 35L1

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value	
Model	374.83	9	41.65	18.27	< 0.0001	significant
A-Temp.	152.22	1	152.22	66.78	< 0.0001	
B-Time	53.48	1	53.48	23.46	0.0007	
C-pH	5.61	1	5.61	2.46	0.1479	
AB	1.89	1	1.89	0.8298	0.3838	
AC	30.77	1	30.77	13.50	0.0043	
BC	21.81	1	21.81	9.57	0.0114	
A^2	9.47	1	9.47	4.15	0.0688	
B^2	7.56	1	7.56	3.32	0.0985	
C^2	82.22	1	82.22	36.07	0.0001	
Residual	22.79	10	2.28			
Lack of Fit	17.90	9	1.99	0.4059	0.8490	not significant
Pure Error	4.90	1	4.90			
Cor Total	397.62	19				
Test for Adequacy						
R^2 = **0.9427,** Adjusted R^2 = **0.8911,** Predicted R^2 = **0.7412,** Adeq Precision = **16.4252**						

F, Fisher's variance ratio; Cor Total, Totals corrected for the mean; P, probability value (P < 0.05, significant at 5% level); R^2 (R squared), Regression model

The results behave logically; when the parameter values increased from the lower limits to the higher limits, the bioethanol yield also increased, reaching the maximum yield at the midpoint of the variable ranges. This result also helps to predict which factors positively and negatively influenced the ethanol yield. The positive sign in front of the terms thus indicates a synergetic effect, while the negative sign indicates an antagonistic effect on the ethanol yield (Equation 3).

This allowed for the development of the following second-order quadratic solution for the production of ethanol (Y) as a function of temperature (A), time (B), and pH (C):

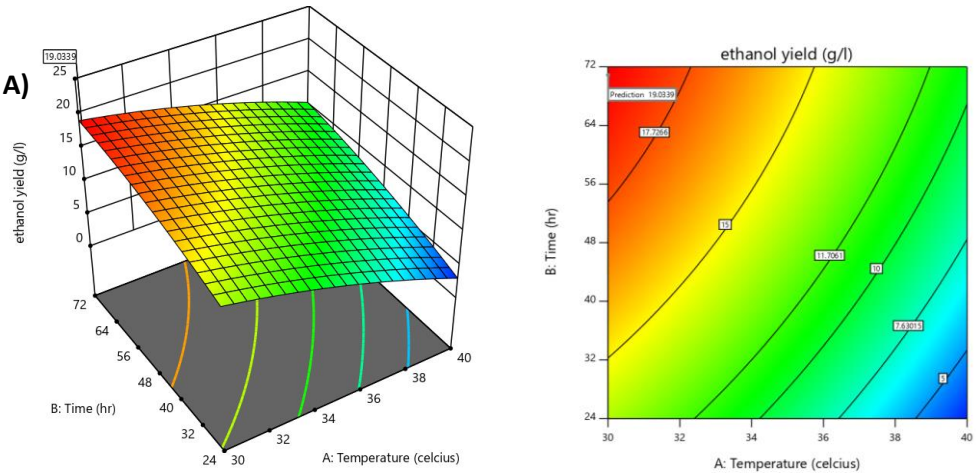
Ethanol yield (Y) = $14.2449 – 3.23A + 1.92B – 0.5983C + 0.4863AB + 1.96AC – 1.65BC – 0.8687A^2 – 0.7832B^2 – 2.67C^2$ ……..……. Equation 3

Where, A, B, AC, BC, and C^2 are significant model terms, as evidenced by the results in Table 5. The degree of significance indicated that the incubation temperature and fermentation time had the most effects on the production of ethanol. Furthermore, variable pH was insignificant in the linear term but highly significant in the quadratic term. This shows that any change in these variables significantly affects ethanol production. Time and temperature together had an insignificant effect ($P > 0.05$).

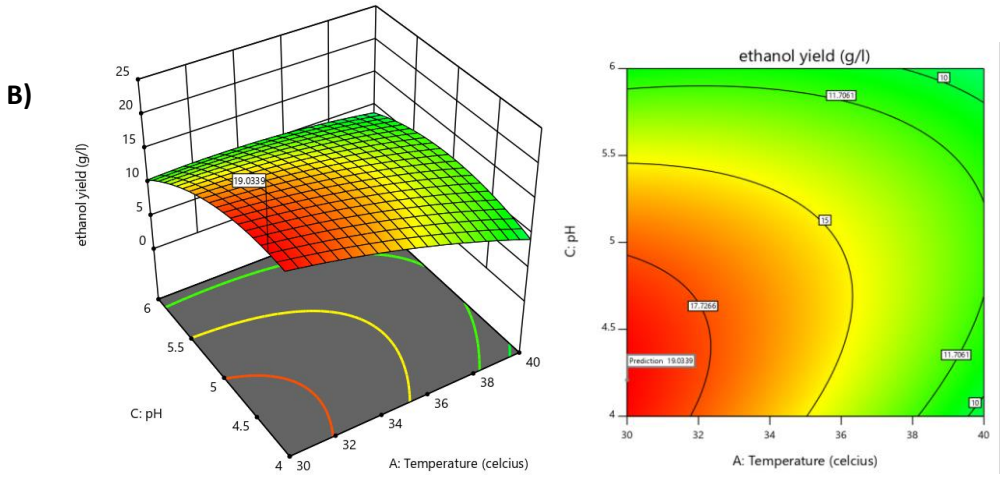
Variables effect on Bioethanol Yield of isolate 35L1

The results shown in Figure 8A demonstrate the relationship between temperature and time in influencing the yield of bioethanol production. Within the temperature range of 30–35 °C and duration of fermentation ranging between 32–72 hours, a high-yield plateau was observed. Similarly, Figure 8B illustrates the impact of incubation temperature and pH on bioethanol production, with the highest yield obtained at a pH range of 4-5.5 and an incubation temperature of 30-35 °C. Furthermore, Figure 8C presents the influence of time and pH on bioethanol yield, indicating that fermentation occurs most effectively within a time frame of 32–72 hours and a pH range of 4.5–5. Overall, the optimal conditions for maximizing bioethanol output involve a temperature of 30.03 °C, a pH of 4.92, and incubation for 71.55 hours.

A)


ethanol yield (g/l)
19.0339
25
20
15
10
5
0
72
64
56
48
40
32
24
B: Time (hr)
30
32
34
36
38
40
A: Temperature (celcius)
ethanol yield (g/l)
Prediction 19.0339
17.7264
15
11.7061
10
7.63083
5
72
64
56
48
40
32
24
B: Time (hr)
30
32
34
36
38
40
A: Temperature (celcius)

B)


ethanol yield (g/l)
25
20
15
10
5
0
19.0339
6
5.5
5
4.5
4
C: pH
30
32
34
36
38
40
A: Temperature (celcius)
ethanol yield (g/l)
11.7061
15
17.7264
Prediction 19.0339
10
6
5.5
5
4.5
4
C: pH
30
32
34
36
38
40
A: Temperature (celcius)

C)

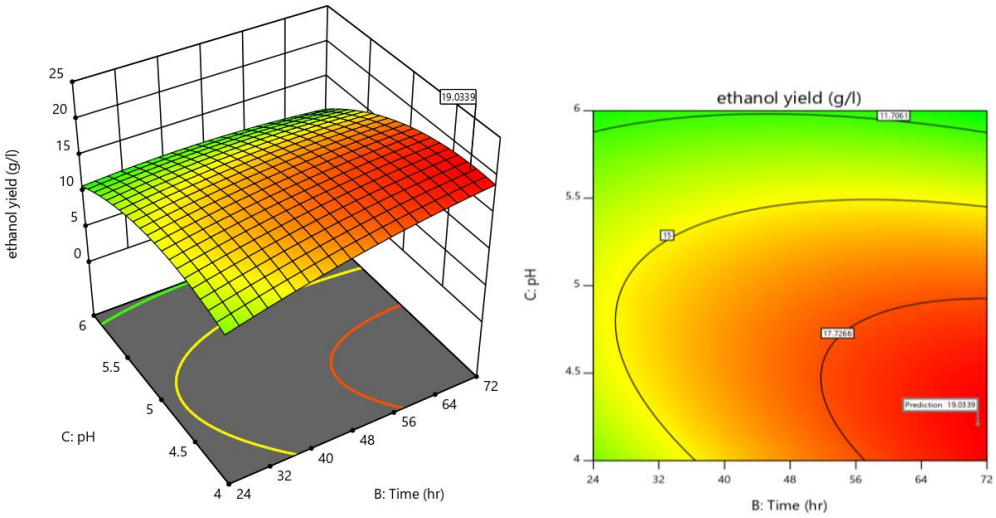

ethanol yield (g/l)
25
20
15
10
5
0
19.0339
6
5.5
5
4.5
4
C: pH
24
32
40
48
56
64
72
B: Time (hr)
ethanol yield (g/l)
11.7061
15
17.7264
Prediction 19.0339
6
5.5
5
4.5
4
C: pH
24
32
40
48
56
64
72
B: Time (hr)

Fig. 8 The response surface (right side) and contour plot (left side) of *S. cerevisiae* 35L1 to pH vs. temperature (A), temperature vs. time (B), and pH vs. time (C) of ethanol (g/L) produced from 40 g/L dextrose.

4. Conclusion

In an effort to overcome the current difficulties in ethanol production, we identified and selected the most efficient yeasts that produced ethanol under stressful conditions. Although the commercial *Saccharomyces cerevisiae* outcompeted the screened bioethanol producing yeast isolates for particular qualities, the test isolates exhibited significant tolerance to higher temperature, low pH, and high concentration of glucose under extended incubation periods. The fact that the *S. cerevisiae* isolate 9Li2 used and vigorously utilized more carbohydrates than the isolate 35L1 and was just as effective at producing ethanol, both *S. cerevisiae* 35L1 and 9Li2 produced the highest amounts of ethanol at dextrose concentration of 120 g/L. The fermentation media's rapid fall in sugar content during the first 24 hours of incubation suggests that *S. cerevisiae* 9Li2 isolate quickly absorbs and uses sugar. For *S. cerevisiae* 9Li2 and 35L1, the ideal pH, temperature, and incubation time were 4.5–5.5, 30-35 ^{o}C, and 48–61 hours; and 4-5.2, 30 ^{o}C, and 48–72 hours, respectively. Traditional optimization techniques for ethanol production are time and resource consuming. The efficiency of the response surface methodology lies in its ability to handle a high number of design parameters while maximizing ethanol yield and lowering production costs. YPD was used in this work to carry out the optimization. If it is done in the actual medium in which ethanol is produced in industry, it is more appropriate. The simultaneous saccharification and fermentation of lignocellulose materials requires the use of microorganisms able to function at high temperatures, along with pentose fermenter yeasts, and this study strongly suggests utilizing these yeasts.

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CRediT authorship contribution statement

TT, Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization; KB: Conceptualization, Formal analysis, Supervision, Review of manuscript, AD: Conceptualization, Formal analysis, supervision, Review of original draft, Visualization, Funding acquisition DD: Investigation. Formal analysis, Writing original draft

Declaration of Competing Interest

The authors declare that they have no competing interests: be it financial and personal.

Data Availability:
The [DATA TYPE] data used to support the findings of this study are included within the article.

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