Research Article

Iranian Grape Waste: A High Potential Source for Screening of Bioethanol-Producing Microorganisms

Robabeh Golestani[1], Masoumeh Mehraban Sang Atash[2], Najme Gord Noshahri[3,*], Mohammad Zare Mehrjerdi[4], Yaser Yousefpoor[5,6]

[1] *Department of Food Science and Technology, ACECR Kashmar Higher Education Institute, Kashmar, Iran*
[2] *Department of Food quality and safety, Academic Center for Education Culture, and Research (ACECR)-Khorasan Razavi Branch, Mashhad, Iran*
[3] *Industrial Microbial Biotechnology Department, Research Institute for Industrial Biotechnology, Academic Center for Education, Culture, and Research (ACECR)-Khorasan Razavi Branch, P.O. Box 91775-1376, Mashhad, Iran*
[4] *Agriculture Faculty of Shirvan, University of Bojnord, Bojnord, Iran*
[5] *Department of Medical Biotechnology, School of Paramedical Science, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran*
[6] *Research Center of Advanced Technologies in Medicine, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran*

Received 18 May 2024 Accepted 31 October 2024

Abstract

The demand for bioethanol as a renewable energy source is rising. This study screened high-ethanol-producing microorganisms found in grapes to reduce production costs and compete with other fuels. The grapes and their waste samples were collected from Iranian vineyards. Microorganisms were initially screened by growing them in a glucose-enriched culture medium containing 10% ethanol to isolate ethanol-tolerant ones. The bioethanol-producing microorganisms were then qualitatively isolated during fermentation using high-throughput screening (HTS) based on CO_2 production and changing bromothymol blue to green color. Promising strains were selected based on the amount of ethanol production by the CO_2 flow meter. The selected strains were identified using 18S rRNA sequencing and PCR by the *S. cerevisiae* species-specific primers (ScHO). Finally, the growth of the most promising strain was optimized using the response surface methodology (RSM) in a shaking incubator. A total of one hundred isolates were tested using HTS devices. Out of these, nine strains were quantitatively screened, and SCL-25 and SCL-62 were chosen to continue based on their high ethanol production rate. The ethanol production rate for SCL-25 was 12.86%, while SCL-62 was 14.35%. After molecular characterization, it was confirmed that SCL-25 was 100% similar to *Saccharomyces cerevisiae*, whereas SCL-62 was 99% similar to *Saccharomyces cerevisiae*. The PCR products amplified by ScHO showed a 400 bp band in agarose gel electrophoresis, confirming them as *S. cerevisiae*. Moreover, isolate SCL-62 showed ethanol production higher than the commercial strain. The RSM optimization results showed that the growth of the SCL-62 strain increased two times at 35 °C, pH 5.0, Brix degree 20, and agitation rate 200 rpm. Therefore, the SCL-62 strain has the potential to produce efficient and cost-effective bioethanol.

***Keywords*:** Bioethanol, high-throughput screening, wild yeast, CO_2 production, grape, response surface method

Introduction

Bioethanol is a green and renewable energy carrier that can be used for biohydrogen generation (Bryant et al., 2020; Palanisamy et al., 2021; Sharma and Sharma, 2021). It is produced through the alcoholic fermentation of fruit, sugar beets, sugar cane, and hydrolysis starches (Jagavati, 2021). Generally, bioethanol production is produced by a type of yeast known as *Saccharomyces cerevisiae* due to its ability to proliferate in low pH environments and its simple nutritional requirements (Nandy and Srivastava, 2018; Walker and Stewart, 2016). This yeast ferments sugar into ethanol and carbon dioxide under anaerobic conditions (Walker and Stewart, 2016).

Strains of bacteria, such as *Zymomonas mobilis* and *Clostridium thermophilic*, some strains of *Acetobacter* and *Lactobacillus* (Kunkee, 1984; Russel, 2003) and yeast can also produce bioethanol. *Ethanol fermentation,* a complex process, poses several challenges. Yeast must withstand high concentrations of sugar and ethanol production stress (Šuranská et al., 2016), as well as inhibitor materials in the medium (Antia et al., 2018). This necessitates industrial yeast to meet specific criteria for efficient fermentation (Greetham et al., 2019).

[*]Corresponding author's e-mail address:
gord.noshahri@jdm.ac.ir

Licensed as Open Access CC-BY

The diversity of microorganisms offers the potential to identify new strains with high ethanol toleration. Several studies have shown that ethanol-producing microorganisms can be isolated from soil, water, plants (Komatsuzaki et al., 2016), and animals.
Furthermore, the initial concentration of microbial cells is another crucial factor affecting ethanol fermentation's efficiency. Cell concentration can be improved through culture optimization. In the past, optimization was carried out using the one-factor-at-a-time approach, which was time-consuming and involved multiple experiments. However, there has been a shift towards using the response surface method (RSM), which reduces the number of experiments required while also allowing for evaluating the interaction between different factors (Bezerra et al., 2008; Nuanpeng et al., 2023).
The results of several research studies show that indigenous strains of organic waste from food and agricultural industries could be enriched sources for screening bacteria and withstand ethanol production stress conditions (AKINRULI et al., 2022; Antia et al., 2018; Hossain et al., 2008). Grapevine, a crop that has stood the test of time as one of the world's oldest and most essential perennial crops (Fatahi et al., 2003), also plays a crucial role in this process. A high soluble sugar content in grapes with TSS above 30% (Duchêne and Schneider, 2005) results in indigenous bacteria and yeasts, contributing to spontaneous alcoholic fermentation (Cappello, Bleve et al. 2004) such as bioethanol. Iranian grapes, with their rich biodiversity and various cultivars grown across 20 provinces (RASOULI, MARVILI et al. 2015), hold significant potential in identifying highly efficient bioethanol-producing microorganisms.
Therefore, this study aims to high-throughput screening of ethanol-producing bacteria from waste grapes using our recently invented devices (Gord Noshahri et al., 2023) and optimize the growth of the most promising strain.

Material and methods

Chemicals

Peptone Yeast Dextrose Agar medium (PDA), glucose powder, yeast extract, sodium chloride, and tryptone powder were purchased from Liofilchem Co., Italy. The Shirvan sugar company in Shirvan, Iran, prepared molasses. Other chemicals and solvents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, US).

Yeast Strain and Culture media

The commercial strain of *Saccharomyces cerevisiae*, used as a control strain, was prepared from Razavi Instant Yeast Co., Iran (SCR) as a reference strain. Modified LB (m-LB) with 0.5% glucose, 0.5% yeast extract, 0.5% sodium chloride, and 1.2% tryptone (pH 6.0) was used as an enrichment medium. After autoclaving, 10% v/v ethanol was added to the m-LB to isolate the ethanol-tolerant microorganisms (Hossain et al., 2018). m-LB agar contains 1.5% agar. A fermentation medium (FEM) consisting of 30% glucose, 1% yeast extract, 0.1% urea, 0.1% ammonium sulfate, 0.1% ammonium phosphate, and pH adjusted 5.5 was used to detect the fermentation characteristics of the strains. The growth medium contains molasses (different brix), 0.5% sodium chloride, 0.1% Urea, 0.1% ammonium sulfate, and 0.1% ammonium phosphate. All percentage values are w/v. Media sterilization occurred at autoclaved (121 ºC, 20 min).

Sampling and enrichment of ethanol-tolerated microorganisms

Two vinegar sediments and 27 samples of grapes were prepared from the grape gardens in Khalilabad (Khorasan Razavi province, Iran). Grapes have a large cultivation area in the Khalilabad region. These grapes were from two Iranian varieties (*Razeghi* and *Asgari*) known for their high yields (Yaghoubi et al., 2016).
Samples of grapes were crushed in a sterile mortar without being washed and poured into sterile containers. Approximately 10 grams of samples were added to 100 ml of m-LB medium (containing 10 v/v% ethanol). Incubation was conducted for seven days in darkness at 30 °C (Figure 1).

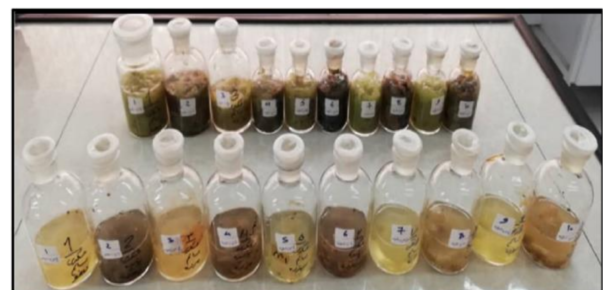

Figure 1. Enrichment of ethanol tolerated microorganisms in LB medium containing 10% ethanol.

Afterward, the culture was diluted in PBS buffer (10:90 µl) and poured onto an m-LB agar plate containing 10% ethanol. The culture was incubated for 48 hours at 30 ˚C. As colonies were visible on the

plate, they were streaked onto a new m-LB agar medium to achieve uniform colonies.

High-throughput screening of ethanol-producing microorganisms

High-throughput screening was performed to identify ethanol-producer microorganisms. The procedure was followed as described by Gord Noshahri *et al.* (Gord Noshahri et al., 2023). Isolated microorganisms were subjected to primary screening: the single colonies were transferred into 10 ml of m-LB and incubated at 30°C with 150 rpm shaking. After overnight incubation, growth was measured using a microplate reader (Epoch Co., USA) at 600 nm. The low-growth bacteria ($OD_{600nm} < 0.3$) were removed from the following process. Each sample was centrifuged (5000×g, 5 min) separately, and the pellet dissolved in saline (NaCl 0.9 w/v %). Cells transferred to FEM medium to adjust OD_{600nm} ~1. Then, 200 µl of each sample was transferred to ODD wells of the 96-well microplate. Next, bromothymol blue reagent (100 µl) was added to EVEN wells. Subsequently, the silicon lid was put on the plate and incubated at 35ºC, 150 rpm. Each experiment was done in triplicate. The commercial strain of *Saccharomyces cerevisiae* (SCR) in FEM medium was applied as a positive control. and FEM medium without inoculum was used as negative control (Figure 2).

The CO_2 flow meter (CFM) device was used as a secondary screening to quantitatively compare the fermentative abilities of the most promising strains (Gord Noshahri et al., 2023). The CFM device is designed to estimate the amount of CO_2 produced during fermentation. According to equations (1) and (2), the volume of CO_2 transferred from the CFM device is converted to ethanol percentage.

A mole of sugar is converted into two moles of ethanol and CO_2 during fermentation. This means that the mole of CO_2 is equal to the mole of ethanol (Ruriani et al., 2012). Therefore, in Equation 2, the mole of CO_2 is used instead of ethanol. Experiments were done in triplicate for each strain. The fermentation condition was 35ºC, 100 rpm.

$$CO_2\,(mole) = \frac{CO_2\,(liter)}{n} \quad (1)$$

$$EtOH\left(\frac{v}{v}\right)\% = \frac{CO_2\,(mole) \times MW}{V} \times 100 \quad (2)$$

Where n is equal to 25.28 (the mole of one liter of gas at 35°C at the atmosphere pressure) according to PV= nRT (Tenny and Cooper, 2017), MW is the molecular weight of ethanol (46.07), and V is the volume of culture medium (liter).

Microscopic and Molecular identification of promising strains

The promising strains were examined using a light microscope with 400× magnification. The highest ethanol-producer strains were chosen for molecular identification. One colony of strains was cultured in a 3-ml of m-LB at 30ºC overnight. One drop of subculture was spread on microscopic slides.

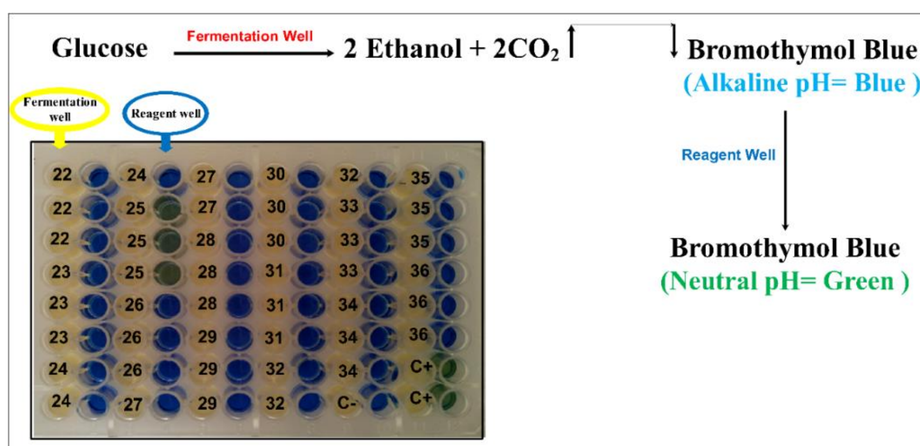

Figure 2. High-throughput screening of ethanol-producing microorganisms by HTS device. The numbers represent the names of the screening isolates. CO2 production of one hundred strains was surveyed in FEM medium. CO2 was transferred via lid to a reagent well-containing bromothymol blue. Then, carbonic acid formed, and the Color changed from blue to green.

It was fixed by heating, and one drop of safranin was added to visualize yeast better. Next, centrifugation (5000 rpm, 5 min) was used to separate the cells. They were diluted with 500 µl of distilled water. Then, genomic DNA was extracted by boiling (Silva et al., 2012). In the following steps, cells were boiled for 10 min. Then, the cell debris was separated by centrifugation (12000 rpm, 5 min). The supernatant was used for the PCR with universal primers (ITS1 and ITS4) to amplify the ribosomal internal transcribed spacer (ITS) region (Table 1). The PCR program was run as follows: denaturation (94 °C, 40 s), annealing (56 °C, 40 s), and extension (72 °C, 60 s) that were repeated in 35 cycles, then the final extension was performed at 72 °C for 10 min. Sanger sequencing was performed by Codon Genetic Group Co. in Iran. The ITS sequence was analyzed using the NCBI BLAST database to identify strains.

Besides, as described by Komatsuzaki et al. (Komatsuzaki et al., 2016), to determine whether local strains are *S. cerevisiae* species or not, specific primers of chromosome 4 (ScHO) are used, as illustrated in Table 1. For the exact determination of whether local strains belong to *S. cerevisiae* species or not, the specific primers of chromosome 4 were used according to Table 1. PCR was done with the following program: 98 ºC for 10 s, 60 ºC for 40 s, and 72 ºC for 60 s (35 cycles), final extension at 72 ºC for 10 min.

Table 1. Primer sequence

Primer	Primer sequence (5ˊ...3ˊ)
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
ScHO- Forward	GTTAGATCCCAGGCGTAGAACAG
ScHO- Reverse	GCGAGTACTGGACCAAATCTTATG

The combined effects of the most influential variables in biomass production of the SCL-62 strain, including ˚Brix, pH, temperature, and agitation rate on biomass production, were investigated by RSM. Several researchers used this method for growth optimization (Gord Noshahri et al., 2021; Hay et al., 2012; Shafaghat et al., 2010). Design Expert 7.0.0 software (Stat-Ease Inc., USA) was applied to design experiments via central composite design. The four factors, including ˚Brix, pH, temperature, and agitation rate, were examined in three levels (+), (0), and (−) in triplicate (Table 2). The software generated 21 experiments with central point: 17.5 ˚Brix, temperature 30˚C, pH 5.5, and 175 rpm agitation rate (Table 3). The response was measured as biomass production (OD_{600nm}) 24 h. In each experiment, the maximum OD_{600nm} was recorded as a response. The point prediction of the Design Expert was applied to obtain the optimum value of the factor for the maximum production level of biomass. The model's accuracy was checked by multiple correlation coefficients (R^2). Then, the predicted optimum condition was done in triplicate and compared with the initial condition (30 ºC, pH 6, 15 ºbrix, 150 rpm).

Table 2. Level of factors used in experimental design

Variables	Symbol	Coded-variable level		
		-1	0	1
ºBrix	A	15	17.5	20
pH	B	5	5.5	6
Agitation rate (rpm)	C	150	175	200
Temperature (ºC)	D	25	30	35

All designed experiments were performed in a 150 ml shake flask with a 30 ml medium. Biomass production was measured at OD_{600nm} and determined as a response. Each run was performed in triplicate.

Results

High-throughput screening of ethanol-producing microorganisms

In this study, one hundred strains were isolated by enrichment culture with ethanol. They were subsequently validated through an HTS device (Figure 2). The HTS colorimetric device indirectly identified bioethanol production by capturing CO_2 in bromothymol blue reagent (Gord Noshahri et al., 2023). The color of bromothymol blue as a pH indicator dye was changed from blue to green due to carbonic acid formation. The HTS result has shown color changing to green in nine strains (SCL-3, SCL-6, SCL-7, SCL-8, SCL-25, SCL-62, SCL-64, SCL-65, and SCL-67) during two hours. Therefore, they can produce bioethanol. However, the speed of color changes in two strains, SCL-25 of the grape and SCL-62 of the vinegar, were more than in others.

Table 3 Design of experiments for the optimization of culture condition for biomass production in SCL-62 strain. Biomass production (OD_{600nm}) was considered as a response.

Std	Run	°Brix	pH	Agitation rate (rpm)	Temperature (°C)	Biomass production (OD_{600nm})
18	1	17.5	5.5	175	30	4.86
1	2	20	6	200	25	3.57
3	3	20	5	200	35	6.83
7	4	15	6	200	35	6.19
9	5	13.2	5.5	175	30	4.25
8	6	15	5	150	25	5.55
10	7	21.7	5.5	175	30	2.67
11	8	17.5	4.6	175	30	5.11
16	9	17.5	5.5	175	38.5	2.75
5	10	20	5	150	35	3.8
15	11	17.5	5.5	175	21.5	1.76
14	12	17.5	5.5	220	30	5.82
4	13	15	6	150	35	4.28
19	14	17.5	5.5	175	30	6.3
2	15	20	6	150	25	2.65
17	16	17.5	5.5	175	30	5.64
21	17	17.5	5.5	175	30	5.52
12	18	17.5	6.3	175	30	4.8
6	19	15	5	200	25	5.07
20	20	17.5	5.5	175	30	5.56
13	21	17.5	5.5	130	30	4.38

The rate of CO_2 production was measured in the promising strains using the CFM device (Figure 3). Results showed that SCL-62 had the highest CO_2 production rate, followed by SCL-25 and SCL-8. SCL-62 also had the highest ethanol production among the tested strains (Table 4), with 13.57% ± 1.64%. On the other hand, SCL-25 produced ethanol at a rate of 12.01%, similar to that of the other screened strains.

Strains identification

The optical microscopic identification confirmed that all nine strains were morphologically similar to the commercial strain (SCR), and are yeast (Figure S1). SCL-62 and SCL-25, the highest ethanol-producer strains, were selected to identify via molecular identification.

PCR by the *S. cerevisiae* species-specific primers (ScHO) of the commercial strain SCL-62 and SCL-25 detected amplified 400 bp. The electrophoresis results confirmed that both strains belong to the species of *Saccharomyces cerevisiae* (Figure S2).

Furthermore, the result of ITS sequencing confirmed that both strains were *Saccharomyces cerevisiae*, SCl-25 (100% similarity), and SCL-62 (99% similarity) (Table 5).

Growth condition optimization by RSM

Table 3 shows the result of biomass production of 21 runs with three repetitions containing five central points The biomass production (OD600nm) was measured after 16 h of culture. The response of the experiments was evaluated in software, and the most appropriate model was selected using analysis of variance. Based on 21 experiments, the cubic model best fits the following equation: A, B, C, and D represent °Brix, pH, agitation rate, and temperature, respectively.

$$\text{Biomass production (600nm)} = +5.40 - 0.47 \times A - 0.092 \times B + 0.40 \times C + 0.29 \times D - 0.24 \times A * B + 0.32 \times A \times C + 0.48 \times A \times D + 0.56 \times C * D - 0.68 \times A^2 - 0.16 \times B^2 - 0.091 \times C^2 - 1.09 \times D^2 - 1.36 \times A \times B \times D + 0.27 \times A^2 \times C - 0.060 \times A \times B^2$$

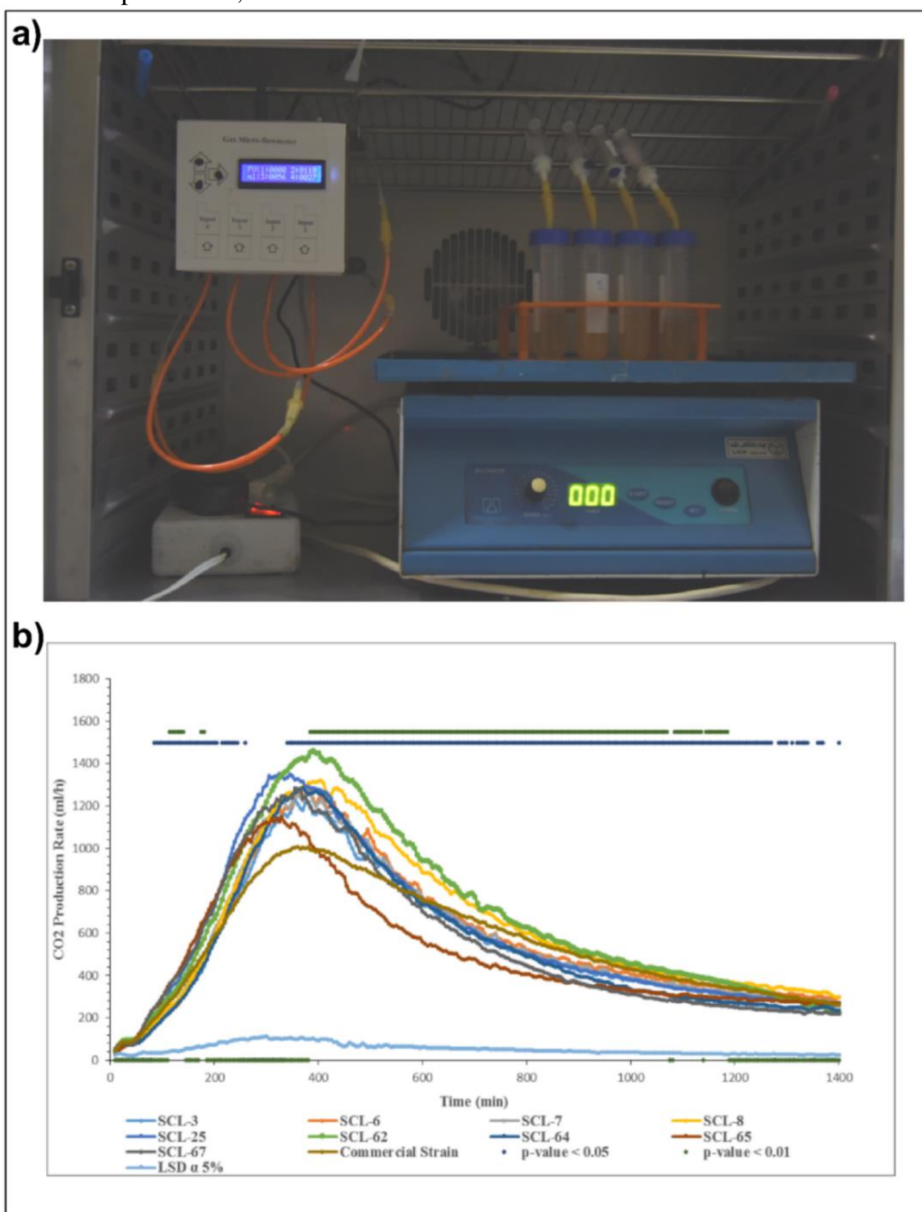

Figure 3. Comparison of CO_2 production rate (ml/h) among screened strains (SCL-3, SCL-6, SCL-7, SCL-8, SCL-25, SCL62, SCL64, SCL65, and SCL-67) and commercial strain (SCR). The experiment was performed as described in the method section for 24h. All experiment was done in triplicate.

Table 4 Comparison of ethanol production (v/v %) based on CO_2 measuring by the CFM device among screened strains and commercial strain (SCR)

Strains	Ethanol (v/v %)
SCL-3	12.56 ±1.18
SCL-6	12.97± 1.16
SCL-7	11.88 ± 0.84
SCL-8	12.91 ± 2.17
SCL-25	12.01 ± 2.62
SCL-62	13.57 ± 1.64
SCL-64	10.96 ± 0.8
SCL-65	10.51 ± 1.1
SCL-67	11.58 ± 1.26
Commercial strain (SCR)	12.7 ± 1.01

After predicting the mathematical relationship, the total validity of the regression average is tested using data analysis of variance indicating the variable's effect (ANOVA analysis, p-value <0.05). Table 6 is a summary of the analyses of variance of regression for biomass production. The R^2 value was 0.98, which indicated the accuracy of the model. The P value is < 0.05 (significant), which shows the model fits well. In this case, A, C, AC, and CD are significant (<0.05) in biomass production, while B, D, AB, and AD are not significant (>0.05). Additionally, the Adeq R-squared, which should be greater than four, was reported to be 6.68, indicating desirability. Figure 4 illustrates a three-dimensional (3)-contour plot of the interaction between two variables. ºBrix and agitation rate were the most influential variables. Observing hot or red spots on the figure increased strain production by increasing ºBrix and agitation rate.

After analyzing and optimizing the test results with Design-Expert software (version 7.0), different factors were provided to achieve the most response. The suggested levels for these factors are a temperature of 20 ºBrix, pH 5.0, 200 rpm, and 35 ºC. Applying optimum conditions, a final OD600nm = 7.31, which agrees with the predicted value (6.83). Comparing the initial condition with OD600nm = 3.8 shows that biomass production increased approximately two times by performing optimization.

Discussion

Global warming, caused by fossil fuel consumption, raises significant ecological concerns. Biofuels, especially bioethanol produced through microbial fermentation, offer a renewable alternative that can power transportation and lessen our reliance on fossil fuels (Maity and Mallick, 2022). Even though the process of alcoholic fermentation has been known for a century, issues still need to be addressed to achieve efficient production, such as the ability of strains to tolerate high ethanol concentrations and yeast cell concentrations (Henderson et al., 2013; Nuanpeng et al., 2023). It is fascinating and valuable to screen and isolate new strains that can produce more ethanol. Fruits with high glucose content provide an optimal growth condition for yeast (Chavan et al., 2009). Various fruits have been screened for this purpose (AKINRULI et al., 2022; Edgardo et al., 2008; Hossain et al., 2018; Khatun et al., 2023; Wang et al., 2022; WIDYANINGRUM et al., 2022), including grapes, which have been studied for their ability to produce ethanol (Favaro et al., 2013; Gronchi et al., 2019; Lu et al., 2020). This study's main objective was to identify and screen microorganisms with high efficiency for ethanol production. In addition, the RSM optimization of parameters for biomass production of SCL-62 was performed in a shaking flask.

The enrichment culture with ethanol (10%) isolated a hundred strains from waste grapes. Growing at 10% (v/v) ethanol showed their tolerance to ethanol, which is needed for the industrial production of bioethanol (Osho, 2005). SCL-25 and SCL-62 were the most promising strains belonging to the *Saccharomyces cerevisiae* species. SCL-62 produced 13.57 v/v% of ethanol, more than the tested commercial yeast (12.7 v/v%).

Table 5. Result of ITS sequencing

Isolate	Percentage of similarity/ Sequence length (bp)	The closest relative in Gene Bank
SCL-25	100/637	Saccharomyces cerevisiae strain B 17 (MN796571.1)[a]
SCL-62	99/633	Saccharomyces cerevisiae strain SB (MK680911.1)[b]
a, b: Accession number in Gene Bank database		

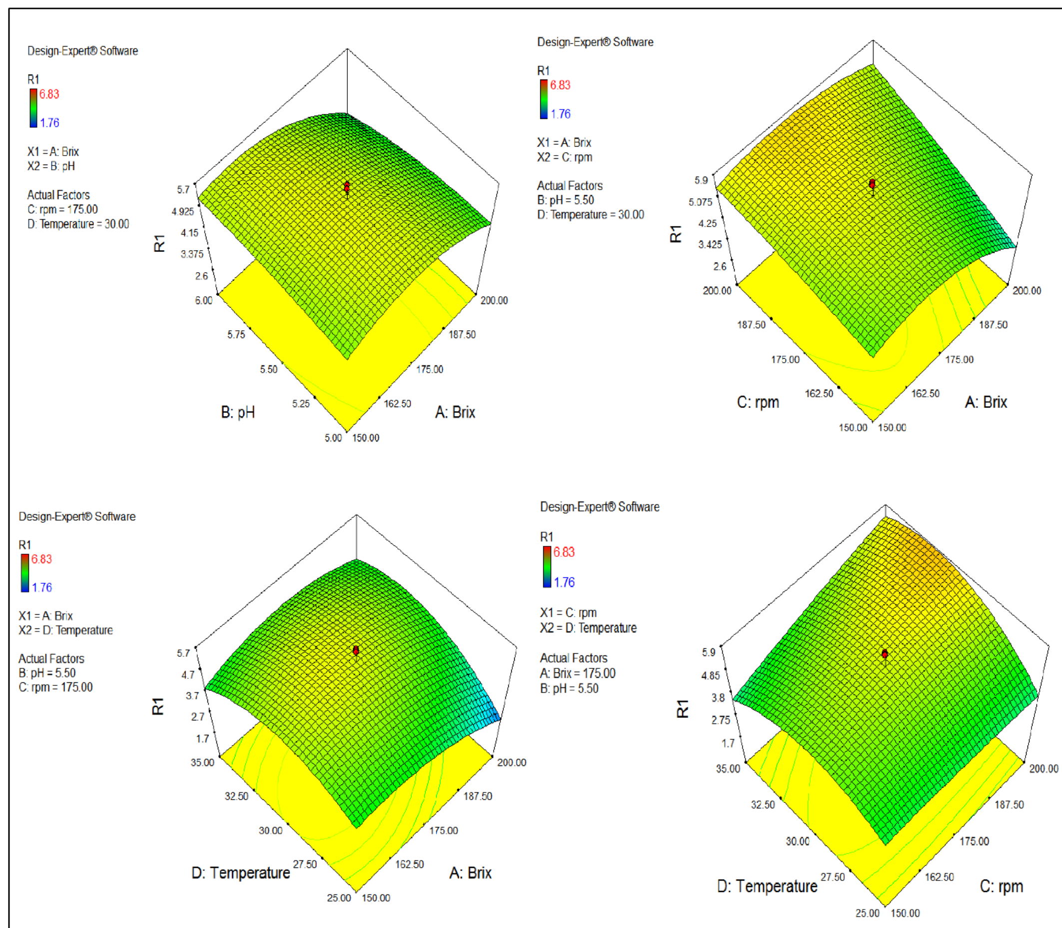

Figure 4. Effect of variables of temperature, °Brix, pH, and agitation rate on maximum biomass production

It has been found that there is a connection between yeast's highest growth and its ability to tolerate ethanol (Henderson et al., 2013). As a result, researchers have conducted many studies to enhance the growth conditions of microorganisms and increase the efficiency of ethanol production, reducing the expenses associated with bioethanol production.

In this study, the RSM software was used to improve the culture conditions in the shake flask. By optimizing the conditions to 20 °Brix, pH 5.0, 200 rpm, and 35°C, it was observed that biomass production doubled compared to the initial condition (15 °brix, pH 6.0, 150 rpm, and 30 °C). The study highlights that the °Brix and agitation rate factors play a significant role in the maximum cell production of SCL-62. In addition, the °Brix factor significantly correlated with the agitation rate and temperature. In general, an increase in °Brix degree leads to a rise in sugar content in the growth medium. However, excessively high °Brix levels can negatively impact microorganism growth, reducing ethanol production due to the high osmotic pressure created by the plasmolysis of cells (Cazetta et al., 2007; Hahn-HäGerdal et al., 1991). Therefore, the ethanol production industry typically tolerates sugar concentrations of less than 200 g/L (Nuanpeng et al., 2023). Tolerance to higher sugar concentrations is required to produce large amounts of ethanol (Osho, 2005).

Ethanol fermentation at high temperatures is necessary in tropical countries with consistently high day-timing temperatures (Banoth et al., 2020). Most of the yeast identified were active at an average growth temperature of 30 °C, which may not be suitable for industrial ethanol fermentation (Bitew et al., 2023; Savitri et al., 2024; Tope et al., 2023). Increasing temperature to a certain level can enhance the growth of living cells. However, if the temperature gets too high, it can reduce membrane phospholipids, damage the cell walls, and denature enzymes (Hahn-HäGerdal et al., 1991; Lee et al., 1981). The SCL-62 strain exhibited optimal growth at 35°C, positively correlated with agitation speed.
According to *Rodmui* et al., increasing the agitation rate to 200 rpm positively impacted achieving a high

biomass concentration (Rodmui et al., 2008). The optimum shaker speed was gained at 200 rpm for the understudy strain. Proper agitation is essential for a culture's physical and chemical conditions to facilitate mixing and mass transfer (Mittal, 1992). The correlation between agitation speed, ºbrix, and temperature was also noted in this study. Increasing the initial concentration of cells can lead to higher ethanol production in the industry (Nuanpeng et al., 2023). As the experimental test demonstrates, SCL-26 shows promise as a strain for bioethanol production in the industry.

Table 6. Analysis of variance (ANOVA) for biomass production

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Probability (P)> F	
Model	33.88	15	2.26	22.65	0.0041	Significant
A- ºBrix	1.25	1	1.25	12.52	0.0241	Significant
B- pH	0.048	1	0.048	0.48	0.5258	Not significant
C- Agitation rate	1.04	1	1.04	10.40	0.0321	Significant
D- Temperature	0.49	1	0.49	4.91	0.0909	Not Significant
AB	0.20	1	0.20	1.96	0.2341	Not significant
AC	0.79	1	0.79	7.96	0.0478	significant
AD	0.76	1	0.76	7.59	0.0511	Not Significant
CD	2.53	1	2.53	25.38	0.0073	significant
A^2	4.99	1	4.99	50.06	0.0021	Significant
B^2	0.26	1	0.26	2.59	0.1829	Not Significant
C^2	0.12	1	0.12	1.16	0.3414	Not Significant
D^2	13.15	1	13.15	131.82	0.0003	Significant
Residual	0.40	4	0.100			
Lack of Fit	9.800E-003	1	9.800E-003	0.076	0.8013	Not significant
Pure Error	0.39	3	0.13			
Cor Total	34.28	19				

Acknowledgments
The authors thank the support of the ACECR Khorasan Razavi Branch, Mashhad.

Funding
The authors thank the support of the ACECR Khorasan Razavi Branch, Mashhad.

Authors' Contributions
NGN and MMSA supervised and designed the experiments. RG and NGN wrote the manuscript. RG and YY performed the experiments. NGN and MZM analyzed the data. All authors read and approved the final manuscript.

Conflict of interest
The authors declare no conflict of interest.

Supplementary figures
Figure S1: Observation of a) SCL-25 and b) SCL-62 using a bright-light microscope at 1000x

magnification. One drop of safranin was added to color the cells red for better visualization.

Figure S2: Result of gel electrophoresis of PCR product with Saccharomyces cerevisiae yeast-specific primer (Scho). NC: Negative control, M: 1kb marker made by Thermo Fisher Scientific, SCR: Commercial strain (SCR), SCL-25, and SCL-62.

References

AKINRULI F. T., IBIYEMI M. F., OLUWASUSI V. O. and AJAYI F. A. (2022) Isolation of microorganisms from cassava peels for the production of bioethanol. GSC Biological and Pharmaceutical Sciences 19:278-287.

Antia U. E., Akan O. D., Stephen N. U., Eno-Ibanga C. K. and Akpan N. G. (2018) Isolation and screening of yeast isolates indigenous palm wine for ethanol production. Philippine Journal of Science 147:411-417.

Banoth C., Kethavath S. N., Keshav P. K. and Bhukya B. (2020) Selection of stress tolerant *Saccharomyces cerevisiae* strains isolated from sloughing off soil for bioethanol production using Prosopis juliflora. Research Journal of Biotechnology 15:1-13.

Bezerra M. A., Santelli R. E., Oliveira E. P., Villar L. S. and Escaleira L. A. (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76:965-977.

Bitew D., Tesfaye A. and Andualem B. (2023) Isolation, screening and identification of ethanol producing yeasts from Ethiopian fermented beverages. Biotechnology Reports 40:e00815.

Bryant S. T., Straker K. and Wrigley C. (2020) Designing our sustainable energy future: A shock doctrine for energy. Energy Policy 147:111914.

Cazetta M., Celligoi M., Buzato J. and Scarmino I. (2007) Fermentation of molasses by Zymomonas mobilis: Effects of temperature and sugar concentration on ethanol production. Bioresource technology 98:2824-2828.

Chavan P., Mane S., Kulkarni G., Shaikh S., Ghormade V., Nerkar D. P., Shouche Y. and Deshpande M. V. (2009) Natural yeast flora of different varieties of grapes used for wine making in India. Food Microbiology 26:801-808.

Duchêne E. and Schneider C. (2005) Grapevine and climatic changes: a glance at the situation in Alsace. Agronomy for sustainable development 25:93-99.

Edgardo A., Carolina P., Manuel R., Juanita F. and Baeza J. (2008) Selection of thermotolerant yeast strains *Saccharomyces cerevisiae* for bioethanol production. Enzyme and Microbial Technology 43:120-123.

Fatahi R., Ebadi A., Bassil N., Mehlenbacher S. and Zamani Z. (2003) Characterization of Iranian grapevine cultivars using microsatellite markers. VITIS-GEILWEILERHOF- 42:185-192.

Favaro L., Basaglia M., Trento A., Van Rensburg E., García-Aparicio M., Van Zyl W. H. and Casella S. (2013) Exploring grape marc as trove for new thermotolerant and inhibitor-tolerant *Saccharomyces cerevisiae* strains for second-generation bioethanol production. Biotechnology for Biofuels 6:1-14.

Gord Noshahri N., Fooladi J., Engel U., Muller D., Kugel M., Gorenflo P., Syldatk C. and Rudat J. (2021) Growth optimization and identification of an ω-transaminase by a novel native PAGE activity staining method in a *Bacillus* sp. strain BaH isolated from Iranian soil. AMB Express 11:1-11.

Gord Noshahri N., Sharifi A., Seyedabadi M., Rudat J. and Zare Mehrjerdi M. (2023) Development of two devices for high-throughput screening of ethanol-producing microorganisms by real-time CO2 production monitoring. Bioprocess and biosystems engineering.

Greetham D., Zaky A. S. and Du C. (2019) Exploring the tolerance of marine yeast to inhibitory compounds for improving bioethanol production. Sustainable Energy & Fuels 3:1545-1553.

Gronchi N., Favaro L., Cagnin L., Brojanigo S., Pizzocchero V., Basaglia M. and Casella S. (2019) Novel yeast strains for the efficient saccharification and fermentation of starchy by-products to bioethanol. Energies 12:714.

17- Hahn-HäGerdal B., Lindén T., Senac T. and Skoog K. (1991) Ethanolic fermentation of pentoses in lignocellulose hydrolysates. Applied Biochemistry and Biotechnology 28:131-144.

18- Hay J. X. W., Wu T. Y., Teh C. Y. and Jahim J. M. (2012) Optimized growth of *Rhodobacter sphaeroides* OU 001 using response surface methodology (RSM).

Henderson C. M., Lozada-Contreras M., Jiranek V., Longo M. L. and Block D. E. (2013) Ethanol production and maximum cell growth are highly correlated with membrane lipid composition during fermentation as determined by lipidomic analysis of 22 *Saccharomyces cerevisiae* strains. Applied and environmental microbiology 79:91-104.

Hossain A., Saleh A., Aishah S., Boyce A., Chowdhury P. and Naqiuddin M. 2008. Bioethanol production from agricultural waste biomass as a renewable bioenergy resource in biomaterials. *In* 4th Kuala Lumpur international conference on biomedical engineering 2008. Springer. 300-305.

Hossain T., Miah A. B., Mahmud S. A. and Mahin A.-A.-. (2018) Enhanced bioethanol production from potato peel waste via consolidated bioprocessing with statistically optimized medium. Applied Biochemistry and Biotechnology 186:425-442.

Jagavati S. (2021) Advances in Bioethanol Production: Processes and Technologies. Bioenergy Research: Commercial Opportunities & Challenges:189.

Khatun F., Islam Z., Habib A., Saha S. and Yasmin S. (2023) Sustainable utilization of fruit wastes for production of bioethanol using thermotolerant *Saccharomyces cerevisiae* yeast isolated from common fruits of bangladesh. journal of animal and plant sciences-JAPS 33:440-452.

Komatsuzaki N., Okumura R., Sakurai M., Ueki Y. and Shima J. (2016) Characteristics of *Saccharomyces cerevisiae* isolated from fruits and humus: Their suitability for bread making.

Kunkee R. (1984) Selection and modification of yeasts and lactic acid bacteria for wine fermentation. Food Microbiology 1:315-332.

Lee K., Skotnicki M., Tribe D. and Rogers P. (1981) The effect of temperature on the kinetics of ethanol production by strains of *Zymomonas mobilis*. Biotechnology Letters 3:291-296.

Lu Y., Sun F., Wang W., Liu Y., Wang J., Sun J., Mu J. and Gao Z. (2020) Effects of spontaneous fermentation on the microorganisms diversity and volatile compounds during 'Marselan'from grape to wine. Lwt 134:110193.

Maity S. and Mallick N. (2022) Trends and advances in sustainable bioethanol production by marine microalgae: A critical review. Journal of Cleaner Production:131153.

Mittal G. S. (1992) Food biotechnology: techniques and applications. (No Title).

Nandy S. K. and Srivastava R. (2018) A review on sustainable yeast biotechnological processes and applications. Microbiological research 207:83-90.

Nuanpeng S., Thanonkeo S., Klanrit P., Yamada M. and Thanonkeo P. (2023) Optimization Conditions for Ethanol Production from Sweet Sorghum Juice by Thermotolerant Yeast *Saccharomyces cerevisiae*: Using a Statistical Experimental Design. Fermentation 9:450.

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

Palanisamy A., Soundarrajan N. and Ramasamy G. (2021) Analysis on production of bioethanol for hydrogen generation. Environmental Science and Pollution Research 28:63690-63705.

Rodmui A., Kongkiattikajorn J. and Dandusitapun Y. (2008) Optimization of agitation conditions for maximum ethanol production by coculture. Agriculture and Natural Resources 42:285-293.

Ruriani E., SUNARTI T. C. and Meryandini A. (2012) Yeast isolation for bioethanol production. HAYATI Journal of Biosciences 19:145-149.

Russel I. 2003. Undserstanding yeast fundamentals, The Alcohol Textbook, Chapter 9, 85-119. Nottingham University Press.

Savitri E. S., Rahmah A. and Daryono R. N. H. 2024. Screening and characterization of potential bioethanol production yeast from tropical fruits. *In* IOP Conference Series: Earth and Environmental Science. Vol. 1312. IOP Publishing. 012037.

Shafaghat H., Najafpour G. D., Rezaei S. P. and Sharifzadeh M. (2010) Optimal growth of Saccharomyces cerevisiae (PTCC 24860) on pretreated molasses for ethanol production: Application of response surface methodology. Chemical Industry and Chemical Engineering Quarterly 16:199-206.

Sharma N. and Sharma N. (2021) Screening and molecular identification of hypercellulase and xylanase-producing microorganisms for bioethanol production. Current Science 120:841.

Silva G. A. d., Bernardi T. L., Schaker P. D. C., Menegotto M. and Valente P. (2012) Rapid yeast DNA extraction by boiling and freeze-thawing without using chemical reagents and DNA

purification. Brazilian Archives of Biology and technology 55:319-327.

Šuranská H., Vránová D. and Omelková J. (2016) Isolation, identification and characterization of regional indigenous *Saccharomyces cerevisiae* strains. brazilian journal of microbiology 47:181-190.

Tenny K. M. and Cooper J. S. (2017) Ideal Gas Behavior.

Tope A. K., Johnson A. O. and Rachael O. T. (2023) Evaluation of *Saccharomyces cerevisiae* Improved Strains Potential in the Bioethanol Production from Bagasse. Biotechnology Journal International 27:16-28.

Walker G. M. and Stewart G. G. (2016) *Saccharomyces cerevisiae* in the production of fermented beverages. Beverages 2:30.

Wang X., Wang Z. and Feng T. (2022) Screening of yeast in various vineyard soil and study on its flavor compounds from brewing grape wine. Molecules 27:512.

WIDYANINGRUM T., FEBRIANTI N., PRASTOWO I., SAIFUDDIN M. F. and PERMADI A. (2022) Exploration, screening and identification of indigenous yeast from some palm juices for bioethanol production. Biodiversitas Journal of Biological Diversity 23.

Yaghoubi A., Tahmasbi A. M., Naserian A. A., Valizadeh R. and Vakili S. A. (2016) A comparison of nutritional value of raisin wastes obtained from two Iranian grape varieties by in vitro rumen fermentation. Direct Research Journal of Agriculture and Food Science 4.

Open Access Statement:
This is an open access article distributed under the Creative Commons Attribution License (CC-BY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Supplementary Figures

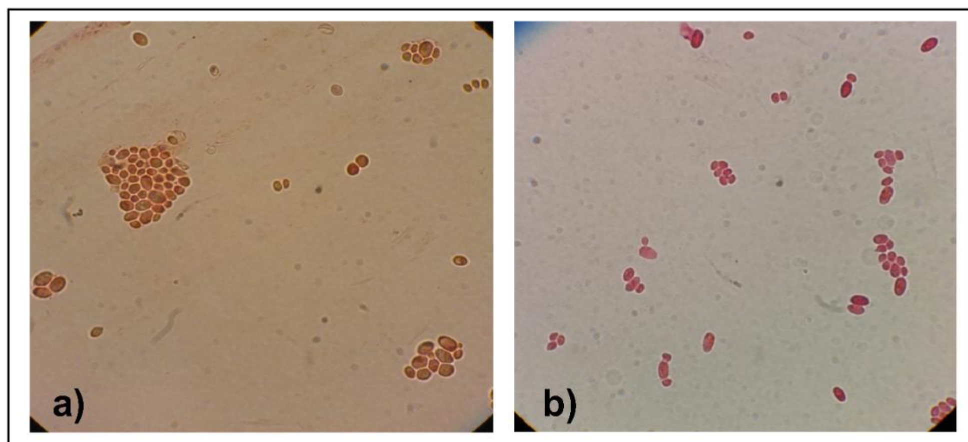

Figure *S1*. Observation of **a)** SCL-25 and **b)** SCL-62 using a bright-light microscope at 1000x magnification. One drop of safranin was added to color the cells red for better visualization.

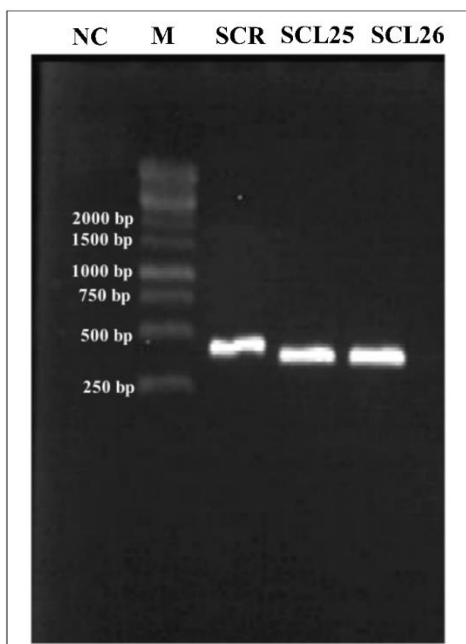

Figure *S2*. Result of gel electrophoresis of PCR product with *Saccharomyces cerevisiae* yeast specific primer (Scho). NC: Negative control, M: 1kb marker made by Thermo Fisher Scientific, SCR: Commercial strain (SCR), SCL-25, and SCL-62.