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The Effect Of Glucose, Temperature And Ph On Bioethanol Production By *Saccharomyces Cerevisiae*

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Abstract

The study used *Saccharomyces cerevisiae* to ferment sugar to produce ethanol, examining its ability to grow and ferment glucose at different temperatures and pH. Temperature, substrate concentration, and pH all impacted ethanol fermentation process. The system showed high rates of ethanol production and cell growth at 30-45°C, with maximum sugar conversion at 30°C. The optimal pH range for ethanol production was 4.0 to 5.0, with the highest specific ethanol production rate at 120 mg/g.h at pH 5.0. the level of oxygen concentration allowed yeast to use ethanol as a carbon source, unlike in the absence of oxygen. The findings demonstrated that *S. cerevisiae* could grow and ferment glucose at high temperatures, however the efficiency of the fermentation dropped as the temperature rose over a specific point. Additionally, up to a certain point, greater substrate concentrations enhanced ethanol production until the yield stop increasing. With an ideal pH range being identified for maximal ethanol production, pH also played a significant role in the fermentation of ethanol. According to these results, *S. cerevisiae*-based ethanol fermentation may be considerably improved by controlling temperature, substrate concentration, and pH.

Keywords *Saccharomyces cerevisiae*, Ethanol, Fermentation, Glucose, Biomass

1. Introduction

The conversion of biomass to ethanol is gaining appeal as a clean alternative to fossil fuels. The solid-state fermentation (SSF) technique combines enzymatic cellulose hydrolysis with sugar fermentation to provide better ethanol yields. However, optimum fermentation and saccharification need circumstances such as pH, temperature, and substrate concentration,

lowering enzyme requirements and improving sustainable energy generation (Gao and Fleet 1988; Hashmi et al. 2017). Biomass energy has environmental advantages in addition to its potential as a sustainable energy source. The process of turning biomass into fuel ethanol reduces greenhouse gas emissions and mitigates the impact of climate change by using agricultural waste and organic resources (Aldiguier et al. 2004; Lugani et al. 2020). Biomass energy has environmental advantages in addition to being useful as a sustainable energy source. The process of turning biomass into fuel ethanol reduces the release of greenhouse gases and mitigates the impact of climate change by using agricultural waste and organic resources (Broda et al. 2022). Furthermore, the incorporation of SSF into biomass conversion technologies not only boosts ethanol yields but also provides a more environmentally friendly and effective approach to biofuel production. Furthermore, broad use of biomass energy may help rural development by producing jobs and fostering economic development in farming areas (Zhao et al. 2015; Lin et al. 2023). These circumstances guarantee efficient cellulose-to-ethanol conversion, making SSF a more environmentally friendly and cost-effective process than different hydrolysis and fermentation. Furthermore, combining fermentation with saccharification in SSF shortens total processing time and streamlines the production workflow, making it a potential solution for large-scale bioethanol production (Zhao et al. 2015; Branco et al. 2019; Murillo-Alvarado and Flores Russell 2022).

Bioethanol synthesis from yeast *Saccharomyces cerevisiae* fermentation is a well-studied and commonly utilized technology. This yeast can convert sugars contained in a range of feedstocks into bioethanol, including sugarcane, wheat, and vegetative material. Due to their ability to ferment a variety of feedstocks into bioethanol, microorganisms provide a flexible and sustainable option for the production of biofuels (De Bari et al. 2014; Sebayang et al. 2016). Microorganisms are also used in fermentation, which is not only effective but also environmentally benign because it emits fewer greenhouse gases than conventional fossil fuels. *Saccharomyces cerevisiae* is another intriguing option for large-scale bioethanol production due to its capacity to endure high ethanol concentrations (Sanni et al. 2022; Ezzat et al. 2023). Batch fermentation, continuous fermentation, simultaneous saccharification and fermentation are all methods used to produce bioethanol. These methods employ *Saccharomyces cerevisiae* to anaerobically ferment glucose into ethanol. Batch fermentation is the process of adding a certain quantity of feedstock and Saccharomyces cerevisiae to a fermenter and allowing the mixture to ferment for a specific period of time. The process of constantly adding substrate and yeast to a fermenter while removing the fermented product is referred to as continuous fermentation (Alriksson et al. 2011; Jeevan Kumar et al. 2020; Tse et al. 2021).

The main challenge with cellulose SSF is the temperature difference between fermentation (25-30°C) and saccharification (40-50°C). To protect yeast viability, the SSF process should be run between 30 and 40°C. Thermotolerant yeasts can convert glucose to ethanol at similar temperatures, but distillation costs are higher at low ethanol concentrations. Researchers are exploring concentrating sugar solutions before fermentation to improve efficiency. Factors like

pH, CO_2, and temperature affect fermentation rate and sugar absorption (Parawira and Tekere 2011; Shen et al. 2012; Singh et al. 2014).

The variation between the optimal temperatures needed for fermentation (30-35°C) and saccharification (40- 45°C) presents the main difficulty with SSF of cellulose. The SSF process is often carried out between 30 and 40°C, far below its maximal working level of cellulose, in order to preserve the life span of the yeast. the application of thermotolerant yeasts, which produce ethanol from glucose at temperatures that are conducive to the cellulolytic complex's function (Ludwig et al. 2013; Karagöz and Özkan 2014). The efficiency of the fermentation system must also be improved in order to use the fermentable sugars that are currently being generated or transformed. However, several studies have looked at the concept of concentrating sugar solutions before distilling them since the distillation cost per unit quantity of ethanol produced is much higher at low ethanol concentrations (Kim et al. 2013; Lin et al. 2014). It is evident that improving the efficiency of fermentation process for ethanol and finding a solution to the issue with the quantity of ethanol produced and glucose supplied are essential if an economically feasible system based on this approach is to be developed. The precise pace of growth during batch fermentation can be significantly impacted by a variety of critical variables, including pH, CO_2, and temperature. Cell number survival, fermentation rate, and sugar absorption rate are all directly influenced by the required medium environment (Chander Kuhad et al. 2010; Tian et al. 2013).

This study aimed to increase ethanol production while maintaining productivity and investigate *Saccharomyces cerevisiae*'s thermotolerance. It examined the effect of temperature, pH level, and starting glucose content on ethanol synthesis by *S. cerevisiae*. The researchers conducted experiments at different temperature and pH levels to determine their impact on ethanol synthesis. They also varied the initial glucose content to observe its effect on fermentation efficiency. The findings from this study can be used to optimize ethanol production processes and enhance the overall performance of *S. cerevisiae* in industrial applications.

2. Material and methods

2.1. Yeast strain and culture conditions

The ATCC strains of *S. cerevisiae* had been used in this investigation. The stock cultures of ATCC strains of *S. cerevisiae* were grown on Yeast Mould (YM) agar medium, which included 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1% glucose, and 1.5% agar (Akaracharanya et al. 2011; Bušić et al. 2018). About 300 ml of medium was autoclaved for 20 min at 121°C, the preculture ATCC strains of *S. cerevisiae* was inoculated in medium in a 500 ml flask and incubate the flask for 24 h in shaking incubator.

2.2. Batch fermentation

The experiment of batch fermentation was carried out in duplicate to check the effect of substrates concentration (carbon source, glucose) and temperature on ethanol production under microaerobic condition. The initial concentration of glucose was used (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 g/L) as a carbon source, stirring at 1.8 Hz and $1g/m^3$ oxygen was provided for *S. cerevisiae* growth. About 500 ml of YM broth medium was prepared in 1000 mL

Erlenmeyer flasks and 3, 5, 7 and 10 % of starting culture was added to the YM medium. The pH of the medium was set on 7 by using NaOH and HCl. The inoculated medium was incubated for 10 days in shaking incubator at 30°C and 20 ml of sample was collected after every 24 h of fermentation to check the glucose concentration and yeast growth.

In second experiment, different temperature range (25, 30, 35, 40, 45, 50°C) was checked to maximum growth of yeast in YM medium for 10 days. The YM medium was prepared in sir different flask and inoculum was added in them and placed in shaking incubator for fermentation. The sample was taken after every 24 h of fermentation to check yeast growth and glucose concentration.

After optimizing the conditions, about 10 L of medium was prepared in a close system, the O_2 and CO_2 exhaustion port was also added in system to maintain their levels. To begin the experiment, a specific amount of inoculated yeast was added to a medium containing varying concentrations of glucose.

2.3. Analytical methods

The study utilized a gas chromatograph to analyze ethanol levels using a 3 m x 2.6 mm glass column filled with polyethyleneglycol, filter samples, measure optical density by spectrophotometer (UV-1600, SHIMADZU) at 600 nm, and determine cell growth rate. The dry weight method was used to determine biomass percentage in suspended solids. Samples were centrifuged, washed, and dried at 105°C for 2 h (Gao and Fleet 1988; Aldiguier et al. 2004).

3. Results

3.1. Temperature and incubation time

In order to determine the best conditions for temperature and choose strains for further investigation, this study examines the influence of elevated temperatures on the biomass and ethanol production of *S. cerevisiae* throughout the ethanol fermentation process. Optimizing the ethanol fermentation processes demands a thorough understanding of how temperature affects biomass and ethanol output. Researchers can increase the efficiency and yield of *S. cerevisiae* strains used in ethanol production by determining the appropriate temperature range. Additionally, this research will aid in the selection of certain strains with greater temperature resilience, opening the door for additional investigation and breakthroughs in the production of biofuel.

The study conducted batch fermentation in shake flasks at different starting glucose concentrations for one week at constant temperatures. The cells expanded rapidly at the start of incubation, but higher temperatures slowed their exponential development. The maximum fermentation period decreased as temperature rose, but cell growth and fermentation significantly decreased at 50°C.

The greater temperature enhanced the transportation process of solvents and soluble compounds in cells, perhaps causing more ethanol to accumulate. The slower specific growth rates might be attributed to the cells' reduced reactivity to ethanol at lower temperatures. With different initial glucose concentrations, the highest specific growth rate and ethanol yield were seen between 30 and 45°C. According to the study, cells growing on glucose initially switched from an oxygen-

dependent metabolism dependent on tricarboxylic acid, glyoxylate, and mitochondrial electron transport chains to a fermentative metabolism based on glycolysis and producing ethanol to break down the ethanol produced during earlier stages of growth.

Furthermore, the indirect impacts of high temperatures may contribute to ribosome and enzyme denaturation, as well as problems with membrane fluidity. Slower specific growth rates might be explained by the cells' decreased tolerance to ethanol at lower temperatures. With different initial glucose concentrations, the maximum specific growth rate and maximum specific ethanol production rate were recorded between 30 and 45°C, as shown in Fig. 3. Conventional knowledge holds that practically all fermentation would be troublesome between 20 and 35°C. Despite the system still showing robust rates of cell growth and ethanol production at 45°C, as demonstrated in Figs. 2 and 3 in our study, and the lowest mt/m30 at varied glucose concentrations.

3.2. Glucose concentration

With an initial glucose concentration of 40 g/m^3 and a yeast concentration of 2 g/m^3 of SS, Fig. 3 shows the fluctuations in ethanol concentration at various temperatures throughout the course of a week of incubation. The greatest fermentation duration, 120 h, was needed for the batch-processed synthesis of general ethanol by yeast. The researchers discovered that higher temperatures inhibited cell growth and fermentation, reducing the maximum fermentation period and ethanol production. This was due to increased soluble chemical and solvent transport activity in cells. The experimental findings are shown in Fig. 3. The maximum fermentation duration reduced as temperature increased, but when temperature was noticeably higher, cell growth was inhibited and fermentation fell drastically. In this work, the inhibitory effect of higher temperatures on cell development was shown by the drastically decreased cell growth and ethanol generation at 50°C.

The experiment involved varying glucose concentrations to develop ethanol production. The substrate concentration influenced ethanol synthesis, and high starting glucose concentrations required longer incubation periods (Fig. 2). Higher initial glucose concentrations may increase ethanol production, but may reduce ethanol conversion efficiency.

The study found that the maximum sugar conversion for different glucose concentrations after 120 h was 35.7%, 46.2%, 18.3%, 14.7%, and 4.6%. Adding more substrate did not increase specific ethanol production rate when pH was not adjusted. The ethanol synthesis was controlled by substrate concentration, and larger initial glucose concentrations may have decreased efficiency when pH was not adjusted.

The ethanol production was controlled by the substrate concentration, which varied from 20 to 200 g/m^3. High starting glucose concentrations above 80 g/m^3 at 30^{o}C required a longer incubation duration when the pH was unregulated, as seen in Fig. 3. It is possible that ethanol production will increase as substrate concentrations rise. If the pH hadn't been adjusted because of the increasing substrate and production, the efficiency of ethanol transformation may have even decreased at higher initial glucose concentrations, like 200 g/m^3. This implies that, particularly at increasing substrate concentrations, pH control is essential for maintaining optimal

ethanol conversion efficiency. The link between substrate concentration and ethanol production may not always follow a linear trend, which highlights the need for more research into the precise processes at play.

3.3. Effect of pH

Due to high concentrations of ethanol and other byproducts generating pH shifts, the ethanol process of fermentation may not take place at greater substrate concentrations. Efficiency may be somewhat increased by adjusting pH. The specific ethanol production rate and fermentation efficiency were considerably improved, when the pH was controlled at 4.0. With beginning glucose concentrations, ethanol fermentation yields improved to 99.8% and 97.4% of the theoretical maximum value after pH adjustment (Fig. 4).

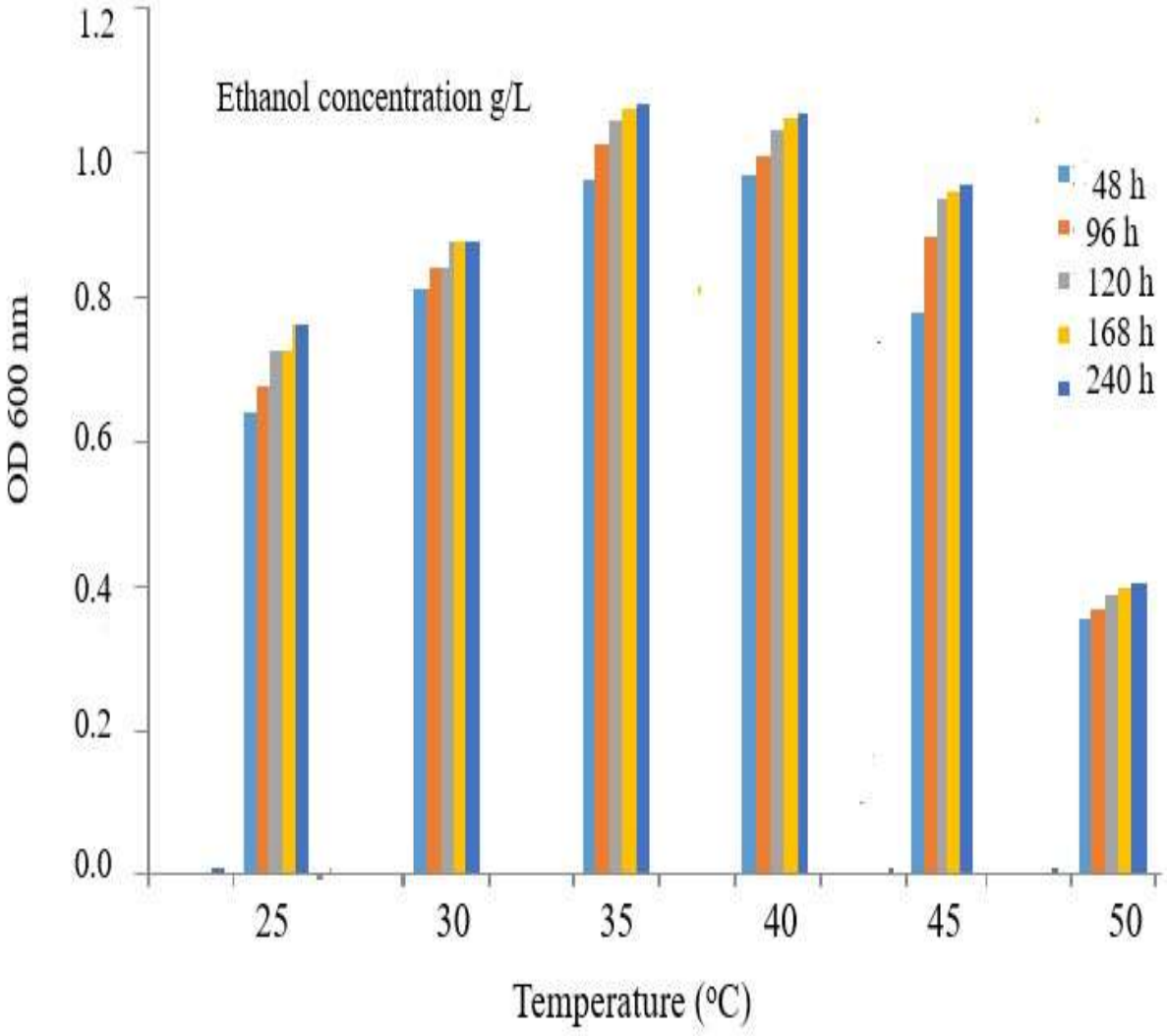

Figure 1. Effect of temperature on the ethanol concentration produced by *S. cerevisiae*.

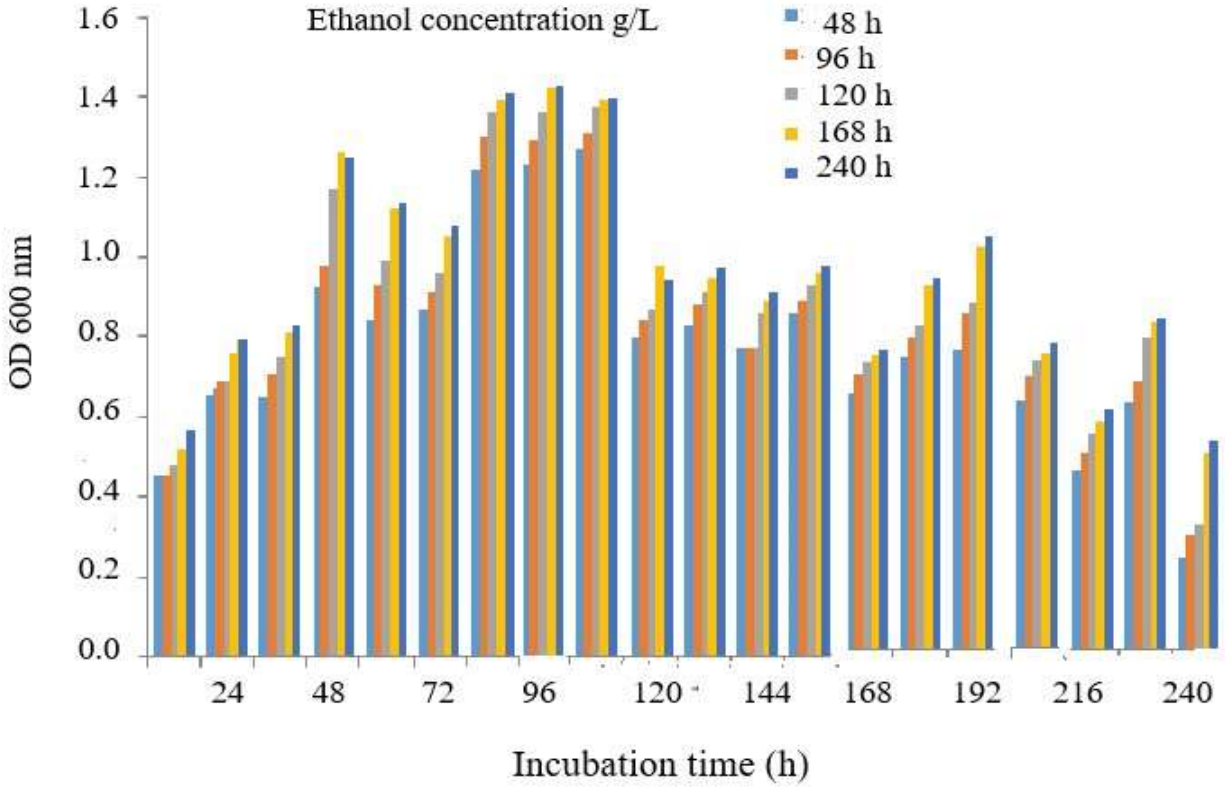

Figure 2. Effect of incubation time on the ethanol concentration produced by *S. cerevisiae.*

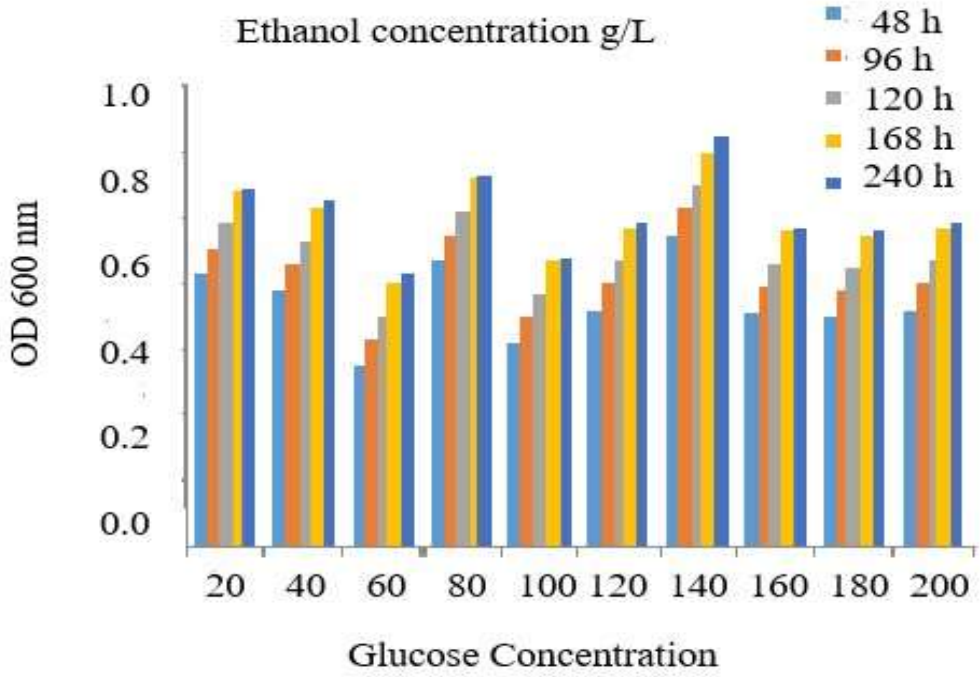

Figure 3. Effect of glucose concentration on the ethanol concentration produced by *S. cerevisiae.*

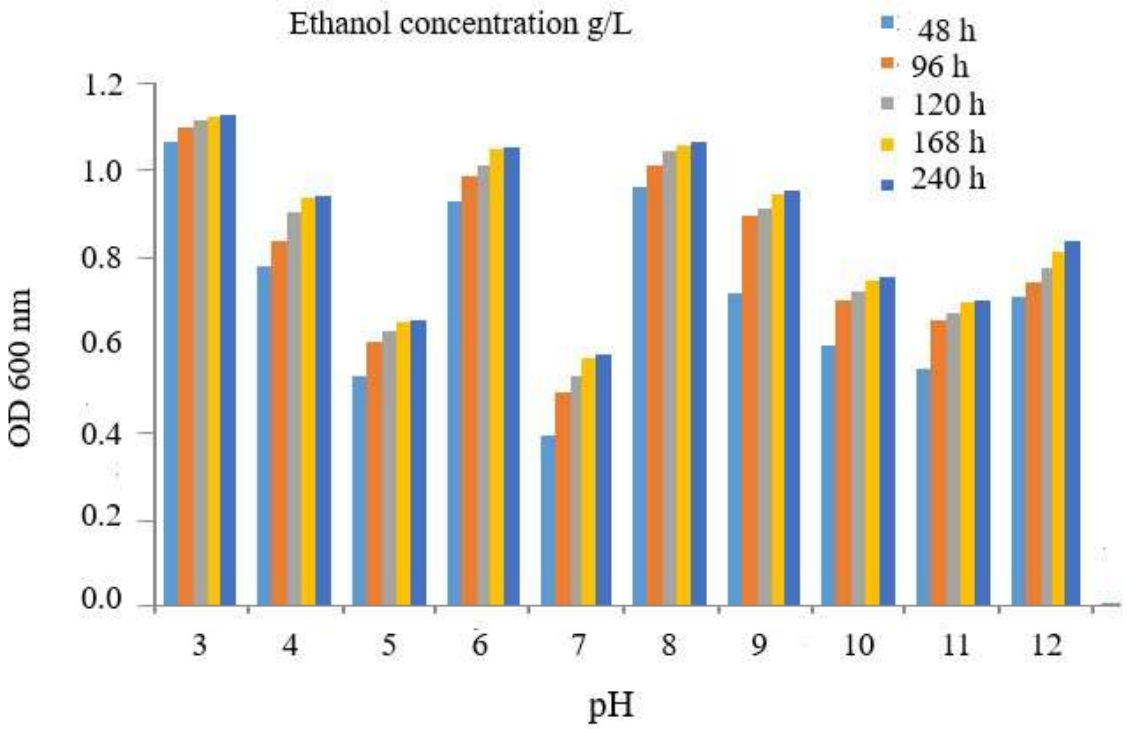

Figure 4. Effect of pH on the ethanol concentration produced by *S. cerevisiae*.

4. Discussion

The capacity of the RND13 yeast strain to produce ethanol at high temperatures and a high substrate concentration [15% (w/v) glucose] near to the level representing industry practice was examined. RND13 is a thermotolerant, fermentative yeast strain isolated from a hot spring drainage. With increasing inoculum size, the RND13 was able to nearly entirely use glucose at 40°C and produce ethanol up to 6.6% (w/v), which is equivalent to values (7.0–7.2%) at 30°C. The RND13 was discovered to produce ethanol at a maximal rate of 9.0 g/L per hour at 40°C in an inoculum sized at 5% (w/v) (Aldiguier et al. 2004). However, at 43°C, the RND13 was unable to completely use the glucose and exhibited a little decrease in the amount of ethanol it generated [6.0% (w/v)]. As a result, it was determined that the culture at 40°C with a 5% cell inoculum was the ideal setting for batch fermentation of ethanol at higher temperatures. The strain was grouped with both *Candida glabrata* and *Kluyveromyces delphensis*, which are quite close to *Saccharomyces cerevisiae*, in the phylogenetic analysis based on the small-subunit rDNA sequencing (Gao and Fleet 1988; Aldiguier et al. 2004).

The ability of the thermotolerant fermentative yeast strain RND13 to produce ethanol at high temperatures was examined. At 40°C, it was discovered to almost entirely consume glucose and produce up to 6.6% ethanol. At 40°C, the greatest rate of ethanol generation was 9.0 g/L per hour. Similar to *Saccharomyces cerevisiae*, the strain was associated with *Candida glabrata* and *Kluyveromyces delphensis*. This shows that the strain used in this study and these closely related yeast species may have genetic traits and metabolic pathways in common. The capacity of the

strain to adapt to and prosper in various environmental situations, such as greater temperatures for ethanol production, may be better understood by understanding these interactions. The precise genetic components that contribute to its special fermentation ability might be investigated in more detail (Ylitervo et al., 2011).

This showed the strain used in the study and these closely related yeast species could share genetic characteristics and metabolic pathways. Understanding these interactions may help us better understand the strain's ability to adapt to and thrive in different environmental circumstances, like as higher temperatures for the manufacture of ethanol. More research may be done to determine the specific genetic elements that contribute to its unique fermentation capacity. Fermentations at higher temperatures were restrictive for growth and ethanol production (Karagöz and Özkan 2014; Lin et al. 2014). Cell viability fell with increasing temperature, with over 90% of viable cells measured until the ethanol concentration reached a threshold value. The best average ethanol productivities remained at about a maximum constant value of 4 g/L.h at 30°C and 33°C. The temperature effect on growth and ethanol production and cell viability remained a significant factor in the fermentation process (Murillo-Alvarado and Flores Russell 2022; Broda et al. 2022b; Sanni et al. 2022).

Various temperatures were used throughout experiments until an uncoupling between growth and ethanol production happened. In the fermentation performances, glucose mass was consumed, and cell and product (ethanol and glycerol) masses were shown vs time. Similar end volumes for the fermentations, with the exception of the one at 39°C, allowed for direct comparison of metabolite concentrations (Tse et al. 2021). Therefore, the interpretation of the fluctuation of the various parameters is unaffected by the dilution factor. At 39°C, the fermentation performed very poorly in terms of growth and ethanol output. The reduced final volume is explained by the fact that just 100 g of glucose were consumed in the span of 27 h and that no more glucose was administered (Jeevan Kumar et al. 2020). As the temperature rose, biomass grew to its highest level at 30°C. The ideal temperature for producing ethanol (approximately 260 g, or 120 g/l) was discovered to be between 30 and 33°C. The three primary by-products were acetic acid, succinic acid, and glycerol. When temperatures were between 27 and 36°C, glycerol production was lower at 30 and 33°C and greater at 36°C, whereas the masses of succinic acid and acetic acid steadily reduced as temperature rose from 10 to 1.9 g and 8 to 1 g, respectively. With the exception of increased glycerol production (11 g), the fermentation remained slow at 39°C, producing very little biomass (2 g), ethanol (43 g), and byproducts (0.7 and 0.5 g of succinic and acetic acid, respectively) (Gao and Fleet 1988; Hashmi et al. 2017). This finding adds to earlier studies that found that glycerol production increased with temperature when *S. cerevisiae* was grown in grape juice in flasks between 15 and 25°C, when mixed yeast was grown on white must in static bottles between 15 and 35°C, or when shochu brewing yeast was exposed to heat shocks between 27°C and 45 to 50°C. Previous research has revealed that when temperature rose, cell viability decreased. Up to a certain level of ethanol concentration, more than 90% of viable cells were observed. Above this point, the cell viability significantly decreased. This ethanol threshold value was discovered to depend on

temperature; at 27°C, it was around 80 g/L, between 30 and 33°C, it was about 100 g/L, and at 36°C, it was about 50 g/L. We saw a sharp decline in cell viability at 39°C just as the fermentation started. The best average ethanol productivities maintained at roughly a maximum constant value of 4 g/L.h at 30°C and 33°C as a result of the temperature influence on growth, ethanol production, and cell survival (Alriksson et al. 2011; Tse et al. 2021).

The study reveals that the sensitivity of yeasts to ethanol is not significantly enhanced by reducing pH to 3.0. The yeasts examined in the study, *K. apiculata* and *C. stellata*, can grow and survive in the presence of ethanol at concentrations up to 9% and 13%, respectively. However, their ethanol tolerances are significantly higher than previously reported values. The study suggests that measurements of ethanol production do not accurately indicate ethanol tolerance, as the majority of ethanol is produced by *S. cerevisiae* in wine fermentation (Karagöz and Özkan 2014; Lin et al. 2014). Therefore, these yeasts have the potential to contribute significantly to low temperature wine fermentation yeast ecology. The impact of pH on yeasts' susceptibility to ethanol has not been well studied, and it is not included in the important sources already listed. It is logical to assume that the cells' susceptibility to ethanol would increase under the extra stress of low pH. However, lowering the pH to 3.0 did not significantly improve the yeasts studied in this study's sensitivity to ethanol as determined by cell viability. To understand the combined effects of low pH and ethanol on the parameters of growth and fermentative activity, more research is necessary. The capacity of *K. apiculata* and *C. stellata* to thrive at ethanol concentrations of up to 9% and 13% and to grow at these concentrations (Karagöz and Özkan 2014; Singh et al. 2014).

5. Conclusions

After studying the effects of temperature, beginning substrate concentration, and pH on ethanol fermentation, the study concluded that *S. cerevisiae* was tested against thermotolerant capacity to grow and ferment glucose at high temperatures. The highest specific ethanol production rates were seen between 30 and 45^{o}C, with ethanol synthesis and cell development occurring more quickly at this temperature. For the most effective ethanol fermentation, the pH must be kept between 4.0 and 5.0.

Data Availability Statement Not applicable.

Conflicts of Interest The authors declare no conflict of interest.

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