DOI: https://doi.org/10.71317/kjard.2.4.2026.733

Kashmir Journal of Academic Research and Development

Journal homepage: https://rjsaonline.org/index.php/KJARD

Enhanced Bioethanol Production by *Saccharomyces Cerevisiae*: Improving Saccharification Efficiency, Fermentation Performance, and Tolerance to Acidic And Osmotic Stress

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ARTICLE INFO

Received:
March 01, 2026
Revised:
March 28, 2026
Accepted:
April 15, 2026
Available Online:
April 25, 2026

Keywords:
Ethanol, *Zymo Faan*, enzyme, *Saccharomyces cervisae.*

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ABSTRACT

The growing demand for renewable energy has driven the development of innovative methods to enhance energy production from sustainable sources. Ethanol production from molasses is a well-established approach that contributes significantly to this effort. In this study, the fermentation process was optimized to maximize ethanol yield by investigating the effect of various operational parameters using the enzyme *Zymo Faan* as an additive. The parameters examined in the study included aeration rate (ranging from 50 to 300 ml/min), agitation speed (100 to 500 rpm), pH (4.0 to 7.0), and temperature (25°C to 45°C). Maximum ethanol production of approximately 86.2 g/l was achieved under the optimized conditions of pH 5.5, temperature 35°C, agitation speed 300 rpm, and an aeration rate of 200 ml/min, using an enzyme dose of 5.0 g/l. Under these conditions, the ethanol yield reached 0.43 g ethanol/g substrate, with a productivity of 1.79 g/l/h over a fermentation period of 48 hours. The maximum ethanol concentration achieved with the addition of 5 g/l of enzyme was 86 g/l, compared to 64 g/l without the enzyme. This represents a 34.4% increase in ethanol production The results demonstrate that the addition of *Zymo Faan* significantly enhances ethanol productivity compared to fermentation without the enzyme. Future research could focus on studying the kinetics of *Zymo Faan* to enable further process optimization and scaling.

Introduction

The rising global demand for sustainable and renewable energy has accelerated research into bioethanol production, positioning it as a viable alternative to fossil fuels in meeting both energy security and environmental goals (2). For developing countries such as Pakistan, the path to socio-economic improvement is strongly linked to adopting reliable and renewable energy sources. Over the past few decades, the world's energy requirements have been met largely by fossil fuels such as coal, oil, and natural gas, while cleaner and more sustainable options were largely overlooked. The extensive use of these conventional fuels has released massive volumes of greenhouse gases, including CO_2 and CO, without adequate concern for environmental consequences (20). In light of worsening environmental pollution, there is now an urgent need to adopt cleaner and more sustainable energy technologies. Moreover, the anticipated socio-economic benefits of conventional energy reliance are proving counterproductive, threatening both environmental balance and societal well-being. At present, fossil fuels account for more than 80% of the global energy supply, nuclear energy contributes about 6%, and the remaining fraction is provided by renewable sources. Among the major energy-consuming sectors, transportation is particularly significant, accounting for around 27% of primary energy use globally, with demand projected to increase by nearly 80% by

2030. In Pakistan, gasoline and related petroleum products represent approximately 40% of current energy consumption, and their use has risen sharply over the past decade, primarily driven by transportation needs (16, 11, 12). This rapid growth in demand has not only increased dependency on fossil fuels but has also led to steep price escalations, burdening both industry and society. Globally, environmental issues linked to industrial pollution are now closely tied to conventional fuel use, underscoring the need for renewable energy alternatives. Ethanol has emerged as one of the most promising substitutes, with commercialization already underway and approximately 3% of global gasoline consumption being replaced by ethanol (3).

Industrial-scale ethanol production relies on the fermentation of hexoses such as sucrose or starch derived from crops like sugarcane, sugar beet, corn, and wheat. A significant share also comes from by-products of sugar processing, such as molasses. Moreover, ethanol derived from cellulosic feedstocks—including agricultural residues, municipal waste, and dedicated energy crops like switchgrass—has gained increasing attention as a sustainable second-generation biofuel (17, 18). In 2011, global sugarcane production reached 1.8×10^9 tons, with Brazil accounting for about 40% of the total, making it the world's leading producer. India, China, Thailand, and Pakistan followed with respective contributions of 19%, 6%, 5%, and 3%. In Pakistan, molasses, a by-product of sugar processing, is the principal raw material for ethanol production. Annual molasses production has ranged from 1.3 to 2.1 million tons over the last decade. Since sucrose loss in molasses impacts process economics, its conversion into ethanol provides a valuable recovery pathway and significantly contributes to the profitability of sugar mills (5). Beyond economic benefits, ethanol production plays a crucial social role in Pakistan by promoting rural development and generating employment opportunities. Against this backdrop, optimizing ethanol yields becomes critically important (13). This study seeks to examine the role of *S cerevisiae*, the widely used fermentative microorganism, in conjunction with Zymo Faan enzyme supplementation, as a strategy for improving ethanol productivity. Specifically, the focus lies on critically assessing the biocatalytic potential of this enzyme-yeast interplay, with the broader aim of enhancing bioethanol yields while supporting the sustainable energy transition in Pakistan and beyond.

Materials And Methods

Microorganism

S cerevisiae was purchased from local market and utilized for ethanol production [14]. A 250 ml production of yeast was prepared, which contained the following constituents (g/l): 10 g glucose, 0.54 g (NH2) HPO4, and 2.4 g yeast extract. The pH was balanced by establishing the medium at 5 and incubating the inoculum at 32 °C and agitating at 160 rpm on an orbital shaker.

Chemicals and growth media

Certain chemicals and growth media are needed for *S. cerevisiae* cultivation to facilitate its development in lab environments. Reagents for the culture of *S. cerevisiae* are usually purchased from reliable vendors like Sigma Chemical Co. 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) NaCl, 0.5% (w/v) glucose, and 2% (w/v) agar make up the growth medium, also known as Sabouraud's medium. For healthy growth, these medium supplies vital nutrients like as carbon from glucose, nitrogen from peptone, and extra salts. To establish the ideal conditions for *S. cerevisiae* culture, 1 M HCl is used to bring the original pH down to 5.5. Before being employed in further research, the inoculum is prepared by cultivating a seed culture on glucose and then rinsing it with sterile saline. A regulated, standardized environment is ensured by these carefully chosen chemicals and growth medium, which encourage robust growth and make it possible to thoroughly examine a variety of facets of *S. cerevisiae* biology and metabolism.

Enzyme *zymo faan*

Zymo Faan is produced by iKnowZyme (online purchase) and is available in both liquid and powder forms. It is designed for application in the fermentation of molasses to increase fermentation efficiency by hydrolyzing the unfermentable sugars. Zymo Faan is a commercially available enzymatic formulation composed of multiple hydrolytic enzymes, with invertase (β-fructofuranosidase), amylases, and cellulases as its primary components. Acting synergistically, these enzymes hydrolyze complex sugars and polysaccharides present in molasses, breaking them down into fermentable monosaccharides such as glucose and fructose. This hydrolysis significantly enhances the availability of sugars for *Saccharomyces cerevisiae* during ethanol fermentation. Notably, the action of Zymo Faan is confined to the substrate rather than the yeast itself, thereby improving fermentation efficiency without exerting lytic effects on the yeast cells.

Although Zymo Faan possesses lysing activity, this function is specifically directed toward plant-derived polysaccharides and structural carbohydrates in molasses rather than targeting yeast cell components. The formulation primarily consists of cellulases, hemicellulases, pectinases, and invertase, which collectively degrade complex carbohydrates into readily fermentable sugars like glucose and fructose. Importantly, Zymo Faan does not contain substantial chitinase or β-1,3-glucanase activity, the enzymes typically associated with compromising yeast cell walls. As a result, *S. cerevisiae* remains

intact and viable throughout the process, ensuring that enzymatic treatment increases substrate availability while preserving yeast functionality and ethanol productivity.

Molasses

Molasses is a dense, viscous liquid that remains after the extraction of sucrose from the mother liquor, which is derived from clarified and concentrated sugarcane or sugar beet juice during sugar production. Blackstrap molasses refers to the final syrup stage, from which further sugar extraction is no longer economically viable. In this study, molasses samples were obtained from Alabbas Sugar Mill Limited and analyzed for their compositional characteristics. The samples exhibited a high Brix level of 91, indicating a highly concentrated solution rich in soluble solids. The sucrose content was 36.10%, highlighting a substantial presence of this key fermentable carbohydrate. Molasses purity was measured at 39.67%, reflecting a balance between desirable sugar components and impurities. The total sugar index (TSI) was 52.56%, while the total sugar (TS) content stood at 50.66%, both indicating the significant presence of various sugars contributing to the overall sweetness. Reducing sugar content was found to be 14.56%, suggesting the presence of sugars capable of undergoing reduction reactions. The net fermentable sugar (NFS) level was 3.5%, indicating the fraction readily available for fermentation. A high fermentable sugar content of 49.06% reinforces molasses' suitability for bioethanol production and other fermentation-based industrial applications, demonstrating its potential as a valuable renewable resource.

Batch-culture studies

In batch culture experiments aimed at microbial ethanol production from molasses using *S cerevisiae*, a yeast salt medium was prepared with the following composition (in g/L): glucose (100), sucrose (150), molasses (170), KH_2PO_4 (5.0), $(NH_4)_2SO_4$ (5.0), $MgSO_4{\cdot}7H_2O$ (0.2), and yeast extract (2.0). Glucose, sucrose and molasses values are simply the stock molasses, used to prepare the fermentation media. After dilution and mixing (and accounting for the actual sugar content of molasses), the **analytically measured initial total fermentable sugar** in the fermentation vessels was ≈120 $g{\cdot}l^{-1}$. The medium pH was adjusted to 5.5. Fermentation was conducted in 23-liter bioreactors maintained at 40 ± 0.2°C. For inoculum preparation, single colonies of *S. cerevisiae* were transferred into 1000-mL Erlenmeyer flasks containing sterile medium supplemented with 10 g/L glucose at pH 5.5. These flasks were incubated for 24 hours on an orbital shaker at 150 rpm and 40°C. Following incubation, the biomass concentration was standardized to 0.84 g/l of dry cell weight before being introduced into the fermentation system (10).Aeration and agitation were maintained at 1.0 vvm and 200 rpm, respectively, with continuous monitoring and regulation of temperature, pH, dissolved oxygen, and gas exhaust. The fermentation medium comprised 15% total reducing sugars (TRS) derived from molasses, supplemented with 10 g/l of $(NH_4)_2SO_4$. The pH was maintained at 5.5 using H_2SO_4. In situ steam sterilization of the medium was performed for 1 hour to ensure aseptic and optimal fermentation conditions.

An active inoculum (10% v/v) was introduced into the fermenter, and automatic pH regulation at 5.5 was achieved using KOH. To promote biomass formation, aeration was initially maintained at 15 l/min for the first 8 hours, after which it was reduced to 3 l/min. pre-autoclaved silicone oil was added as an anti-foaming agent during this transition to control excessive foaming. All optimization experiments were performed in duplicate fermentation. Temperature-dependent profiles of invertase activity and ethanol production were closely monitored. Triplicate time-course samples were collected for analytical assessments. The inoculum medium consisted of 250 ml of sterile solution containing 10 g/l glucose, 0.64 g/l $(NH_4)_2HPO_4$, and 2.5 g/l yeast extract, with the pH adjusted to 5.5. Inoculum cultures were incubated at 30°C for 18 hours with shaking at 160 rpm on an orbital shaker. All media components were prepared using analytical-grade reagents procured from local suppliers.

Determination of kinetic parameters

To investigate the kinetic parameters of batch fermentation, the methodology adopted was based on established protocols from previous studies. The empirical Arrhenius approach was employed to elucidate the relationship between temperature and both microbial growth and ethanol production. Plotting the natural logarithm of cell concentration [ln(X)] against time during the exponential growth phase allowed for the determination of the specific growth rate (μ). Using specific equations specified in the experimental design, growth kinetic constants were computed. The work offered a thorough grasp of the dynamic behavior of biomass accumulation and product creation throughout the fermentation process by utilizing the Arrhenius model, which yielded important insights into the temperature dependency of microbial metabolism.

$(Yp/x) = dP/dx$

$(Yp/s) = dP/ds$

$(qp) = m \times Yp/x$

$(qs) = m \times /Yx/s$

The activation energy needs for cell growth, cell death, product production, and product inactivation were determined in this work using the specific rates of growth (μ) and product formation (qp, g/g cells·h). One important metric that describes how sensitive these biological responses are to temperature changes is the activation energy. The investigation's goal was to measure the energy requirements for each kinetic activity by using the precise rates and techniques described in earlier studies. This method improves our comprehension of the heat impacts on microbial fermentation and the related kinetics by offering insightful information about the temperature-dependent behavior of microbial growth, cell death, and product dynamics.

Methodology

As shown in Figure 01, ethanol fermentation was conducted under control conditions in a 1-l laboratory-scale fermenter. To find the ideal fermentation conditions, several variables were changed, including temperature, pH, aeration rate, and agitation speed (rpm). Additionally, different enzyme doses of *Zymo Faan* were tested to identify the dose that maximizes ethanol production. In the first stage of the experiment, molasses and water were mixed to achieve a 15° Brix solution, and yeast was inoculated along with varying compositions of nitrogen and carbon sources. Enzyme doses were added after a 12-hour incubation period. Three different enzyme concentrations were tested: 2 g/l in the first experiment, 3 g/l in the second experiment, and 5 g/l in the final experiment.



Fig 01 laboratory fermenter

Results And Discussion

Effect of *Zymo Faan* dose on cell mass, substrate utilization and ethanol production

Zymo Faan is an enzyme preparation applied to non-fermentable sugars, preventing their diversion into undesirable by-products during fermentation. Instead, it transforms these sugars into fermentable monosaccharides, which are rapidly converted into ethanol by yeast. In addition to enhancing sugar utilization, Zymo Faan hydrolyzes proteins in molasses, thereby increasing the concentration of free alpha-amino nitrogen (FAN) in the fermentation system. The availability of FAN is particularly critical during the early stages of fermentation, as it promotes yeast growth, accelerates biomass formation, and supports sustained metabolic activity. Furthermore, Zymo Faan exhibits lysing capabilities that help reduce microbial contamination within the system, minimizing acid formation from bacterial growth and thereby favoring ethanol production. These multiple functions collectively explain the positive impact of Zymo Faan on fermentation efficiency observed in the present study.

As shown in Figure 02, the addition of Zymo Faan significantly stimulated yeast growth, with the 5 g/l dose (green line) leading to the highest cell mass accumulation compared to the lower doses (2 g/l and 3 g/l; black and red lines, respectively). This observation can be attributed to the dual effect of increased FAN availability and the enhanced supply of readily fermentable sugars, both of which improve yeast biomass yield. Similar findings have been reported in recent work by Wang et al 2024, who showed that relatively high concentrations of K^+ and Ca^{2+} significantly enhance yeast growth and ethanol yields in concentrated sugarcane molasses, likely by improving osmotolerance and nutrient uptake (24)

Figure 03 further demonstrates that ethanol production was markedly influenced by enzyme concentration. The 5 g/l treatment achieved the highest ethanol concentration (nearly 90 g/l) by the end of fermentation, whereas the 2 g/l and 3 g/l doses produced lower yields. These findings align with prior studies showing that improved substrate conversion and reduced inhibitory by-products lead to faster ethanol accumulation (e.g., Optimization of bioethanol production from sugarcane

molasses by response surface methodology using Meyerozyma caribbica isolate MJTm3 (2023)) where optimized conditions (substrate, time, pH) yielded significantly better performance(9)

Figure 04 depicts the sugar consumption profiles, where the 5 g/l treatment enabled near-complete sugar depletion by 56 hours, compared to the slower utilization observed with lower doses. Faster sugar depletion under higher enzyme concentrations indicates more efficient substrate conversion and improved yeast performance. This is consistent with what has been observed in molasses fermentations where optimized nutrient/cofactor supplementation or adjusted environmental conditions significantly speed sugar uptake and ethanol production (e.g., Key role of K^+ and Ca^{2+} in high-yield ethanol production by *S. cerevisiae* from concentrated sugarcane molasses, 2024)(24).

Taken together, these results demonstrate that Zymo Faan enhances ethanol production by simultaneously improving sugar availability, providing essential nutrients for yeast, and mitigating microbial interference. Such multi-functional benefits highlight the potential of enzyme-assisted fermentation strategies for maximizing ethanol yields. The observed improvements are in agreement with recent trends in the field (2022-2024) where adjusting enzyme dose, optimizing nutrient levels (FAN, minerals), or modulating the fermentation environment (pH, co-factors) have been shown to produce higher yields, faster fermentation, and less by-product formation.

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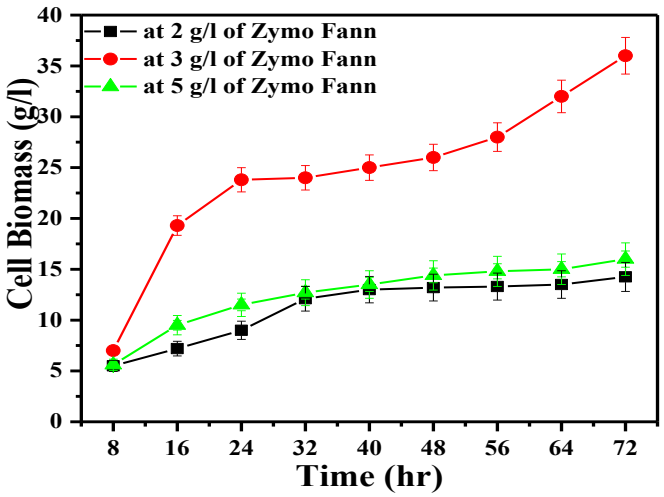


Fig.2. Effect of different doses of enzyme on cell mass production

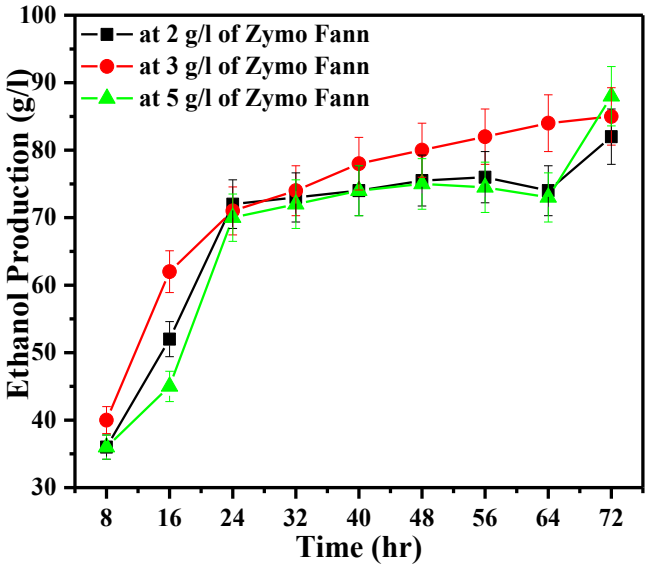


Fig.3. Effect of different dose of enzyme Zymo Fann on ethanol production

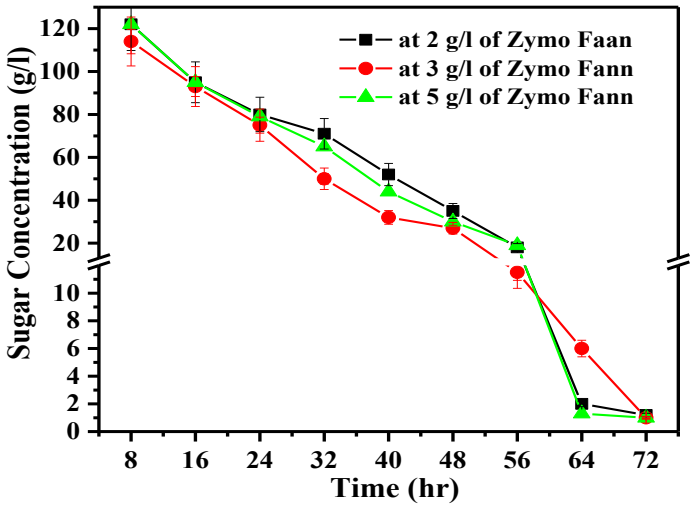


Fig.4. Effect of different doses of enzyme on substrate utlization

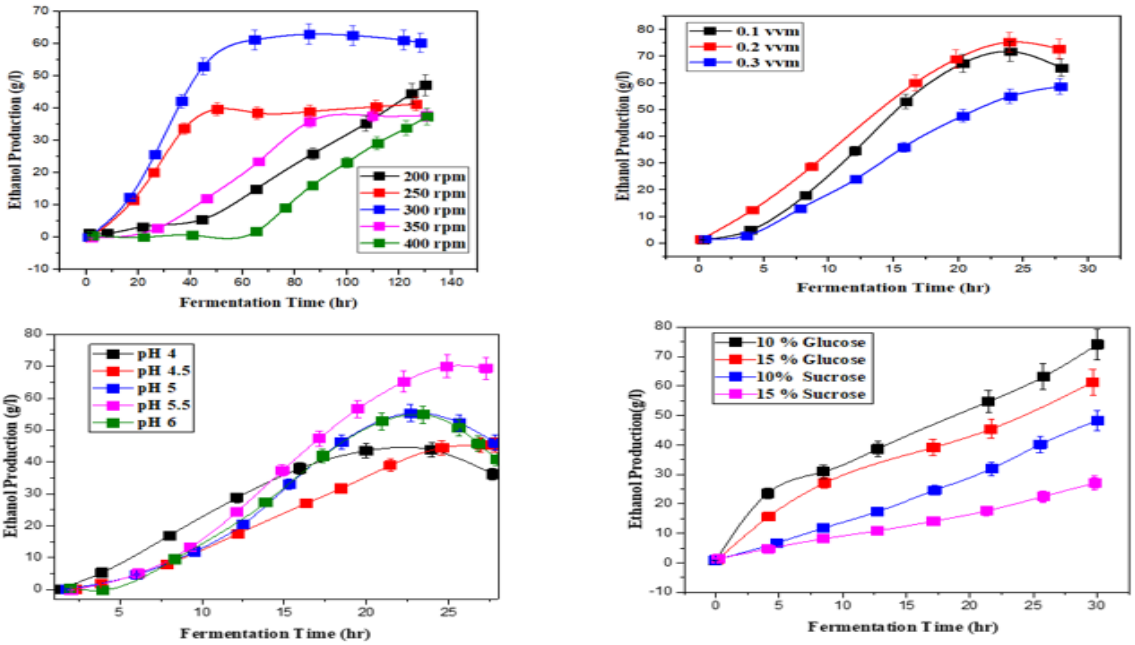
Effect of different parameters on cell mass, substrate utilization and ethanol production

Ethanol production is gaining global interest as a promising alternative energy source, with research focusing on optimizing fermentation processes to improve yields and process efficiency. During fermentation, yeast converts sugars in molasses into ethanol, and several environmental parameters—such as temperature, aeration, pH, and agitation—play decisive roles in shaping fermentation outcomes. *S cerevisiae* remains the most widely used microorganism for ethanol production due to its high ethanol tolerance and efficient sugar utilization capacity, especially with feedstocks rich in fermentable sugars (19, 15, 14). In this study, molasses served as the substrate, while Zymo Faan was used as a biocatalyst to enhance sugar conversion and nitrogen availability.

Figure 05 illustrates the effect of temperature on ethanol production using 5 g/l of Zymo Faan. Ethanol yields were strongly dependent on temperature, with the maximum ethanol concentration (approximately 84 g/l) achieved at 35 °C. At 30 °C and 45 °C, ethanol yields dropped to around 76 g/l and 78 g/l, respectively. These findings are consistent with recent studies reporting that optimal fermentation temperatures for *S. cerevisiae* range from 32 °C to 36 °C, where enzymatic activities and yeast metabolism are most efficient (21). Notably, maintaining fermentation at slightly higher temperatures (>35 °C) can reduce cooling costs, which is attractive for large-scale operations, though careful control is required to prevent stress-induced decreases in ethanol yield (22).

Agitation speed was another critical factor influencing ethanol productivity. Agitation ensures proper mixing, heat dissipation, and nutrient distribution within the fermenter. In this study, agitation speeds ranging from 250 to 400 rpm were tested, with 300 rpm producing the highest ethanol yield (83 g/l). Ethanol concentrations were marginally lower at 250 and 350 rpm (79 and 78 g/l, respectively). This is consistent with research by (23, 1), which showed that mild agitation improves oxygen transfer and keeps yeast viable without resulting in shear stress.

Figure 06 looks at the impact of aeration rate and pH. The findings demonstrated that the pH range of 5.0 to 5.5, which is ideal for yeast growth and metabolic activity, was where ethanol productivity peaked (6). Ethanol yields declined at both higher and lower pH levels, most likely as a result of decreased yeast stability and decreased enzyme activity. Aeration was also essential; at an aeration rate of 200 mL/min, the greatest ethanol concentration (83 g/l) was recorded. During the early growth phase, controlled aeration guarantees enough oxygen for the synthesis of sterol and unsaturated fatty acids, allowing anaerobic metabolism to take over the generation of ethanol. (8) published similar findings, demonstrating that limited aeration balances yeast biomass growth with ethanol buildup to maximize ethanol production. When combined, these results emphasize how crucial it is to maximize ethanol generation from molasses through enzyme-assisted fermentation by adjusting temperature, agitation, pH, and aeration. Recent findings (24, 4, 7, 14) provide strong support for the optimal values found in this study, highlighting how reliable these conditions are for attaining high ethanol productivity in industrial applications.



Challenges and Future Prospects

The study of ethanol production, which includes a variety of substrates and process conditions, presents a number of difficulties as well as exciting opportunities for further research. The complex simultaneous optimization of several parameters presents an inherent difficulty that necessitates a sophisticated comprehension of the interplay between variables like as substrate type, pH, temperature, and carbon source concentration. Despite being instructive, the traditional one-parameter-at-a-time method might not adequately account for any trade-offs or synergies across variables. Developing a reliable and scalable fermentation process is another major obstacle. The switch to larger industrial-scale fermenters adds more complications, requiring additional adjustments for ideal conditions and sustained productivity, even though the knowledge obtained from the study using a 1-liter fermenter is useful for comprehending growth kinetics and ethanol production. Genetic variety is introduced by the focus on mutant strains of Saccharomyces cerevisiae, which presents benefits as well as obstacles. Even though mutants might have improved qualities, it's important to make sure these traits hold up throughout long fermentation times and in a variety of environmental settings. Understanding the genetic processes that underlie enhanced ethanol production may lead to developments in synthetic biology and genetic engineering. In the future, there may be opportunities to investigate cutting-edge fermentation tactics and creative bioprocessing methods. To increase ethanol production and improve substrate utilization efficiency, fed batch or continuous fermentation techniques should be investigated. It is also essential to expand the study to evaluate the optimized ethanol production process's environmental sustainability and economic viability. Product purification and by-product minimization issues could be resolved with a thorough examination of downstream processing and product recovery techniques. Exploring the co-production of value-added compounds alongside ethanol could further bolster the overall economic viability of the process. Given the global emphasis on sustainable practices, future research may delve into the environmental impact and life cycle assessment of the ethanol production process outlined in the study. This holistic approach could provide critical insights into the ecological footprint of the process, guiding efforts toward more sustainable and eco-friendly bioethanol production. In summary, while the study provides valuable insights into ethanol production optimization, overcoming challenges related to process scalability, genetic stability, downstream processing, and sustainability considerations will be pivotal for fully realizing the research's industrial applications.

Conclusion

This study contributes to the advancement of renewable energy by enhancing ethanol production through the strategic use of enzymes. The maximum ethanol concentration achieved was approximately 86 g/l under optimized conditions: pH 5.5, temperature 35 °C, agitation speed 300 rpm, and an aeration rate of 200 ml/min, with an enzyme dosage of 5 g/l. the maximum ethanol concentration achieved with the addition of 5 g/l of enzyme was 86 g/l, compared to 64 g/l without the enzyme. This represents a 34.4% increase in ethanol production. These results indicate that the addition of enzymes to molasses significantly improves ethanol productivity compared to processes without enzymatic supplementation. The enzyme Zymo Faan demonstrated a substantial impact on fermentation performance, and future work should focus on a detailed kinetic study of this enzyme to better understand its behavior and optimization potential. Zymo Faan a complex of enzymes

naturally present in yeast cells, plays a critical role in catalyzing the conversion of fermentable sugars into ethanol via glycolysis and alcoholic fermentation pathways. The synergistic interaction between Zymo Faan and *S cerevisiae* was found to significantly enhance ethanol production. This cooperation leads to faster sugar metabolism, higher ethanol yields, and improved overall fermentation efficiency. Zymo Faan accelerates the rate of biochemical reactions, thereby boosting the performance of *S cerevisiae* as a biocatalyst. The combined effect results in a robust and highly productive bioconversion system, making this approach a promising strategy for industrial-scale ethanol production.

In conclusion, this study highlights the crucial role of enzyme-microorganism interactions in bioethanol production. Optimizing this synergy not only improves fermentation performance but also contributes to the development of more efficient and sustainable biofuel technologies. Future research should aim to elucidate the kinetic mechanisms and molecular dynamics of enzyme action within the fermentation process, paving the way for further innovation in renewable energy production.

Acknowledgement

Thanks for the support of the chemical engineering department, Dawood university of Engineering and technology, Karachi.

Declarations

Funding

N/A

Author contribution

All authors do write, review the paper

Data availability

Available on request.

Ethical approval

Not applicable.

Conflict of interest

The authors declare no competing interests.

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