Journal of Agriculture Sustainability and Environment
https://doi.org/10.56556/jase.v5i1.1413
ISSN: 2997-271X

RESEARCH ARTICLE

Sustainable Utilization of Agricultural Sugar Substrates for Bioethanol Production

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Received: 08 February, 2026, Accepted: 15 April, 2026, Published: 25 June, 2026

Abstract
Optimization of fermentation conditions is essential for the sustainable conversion of agricultural sugar resources into bioethanol. In this study, the effects of pH and urea concentration on ethanol production from two agro-derived substrates, molasses and date juice, were evaluated using Saccharomyces cerevisiae over a 9-day fermentation period. Fermentation was conducted at pH levels ranging from 3.5 to 5.5 and urea concentrations of 0.25–1.0% (w/v), with ethanol concentration monitored daily. The highest ethanol yields were obtained at pH 4.5, reaching 9.4% (v/v) for molasses and 9.3% (v/v) for date juice, followed by pH 4.0. Two-way ANOVA revealed significant effects of substrate type and fermentation duration ($p < 0.001$), while their interaction was not significant. Urea supplementation at 0.5% (w/v) resulted in maximum ethanol production (10.0% (v/v) for molasses and 9.6% (v/v) for date juice), whereas higher concentrations did not enhance yields and, in some cases, reduced final ethanol levels. Ethanol production increased rapidly during the first 5–6 days and subsequently plateaued. These findings demonstrate that optimizing pH and nitrogen input can enhance bioethanol production from agricultural sugar substrates, supporting sustainable waste valorization and efficient bioenergy generation within agricultural systems.

Keywords: Saccharomyces cerevisiae; bioethanol; agricultural by-products; molasses; date juice

1. Introduction

From a broader sustainability perspective, valorizing sugar-rich agricultural by-products into bioethanol supports decarbonization pathways by reducing reliance on fossil-based primary energy and contributing to lower CO_2 emissions, aligning with recent discussions on technology, energy demand, and development trajectories(Etesami et al., 2024; Alihosseini &Hosseini, 2012). Ethanol is a leading renewable biofuel, widely used as a clean additive or alternative to fossil fuels(Tesfaw & Assefa, 2014). Its production via fermentation has grown substantially in recent decades as countries seek sustainable energy solutions(El-Araby, 2024; Chehreara et al., 2022). Saccharomyces cerevisiae is the preferred microorganism for industrial bioethanol fermentation, owing to its high ethanol tolerance, rapid sugar fermentation, and robustness in various conditions(da Silva Fernandes et al., 2022). Conventional feedstocks for ethanol include sugarcane juice, corn starch, and other edible crops, but reliance on food-based substrates raises economic and ethical concerns. To reduce costs and avoid food–fuel competition,

current research is increasingly focused on low-cost, non-food substrates such as agricultural by-products and waste materials(Murugesan et al., 2019). Utilizing these alternative feedstocks not only cuts raw material expenses but also adds value to agricultural residues by reducing waste disposal needs(Tesfaw & Assefa, 2014). Date fruit juice (syrup) and cane molasses are attractive fermentation media due to their high fermentable sugar content and year-round availability. Date palms are extensively cultivated in arid regions, and large quantities of dates are produced annually (e.g. over 1.2 million tons in Iran)(Castillo et al., 2023; Alihosseini, 2015)). A significant fraction of harvested dates are low-grade or unsalable fruits, which can be converted into fermentable "date juice" for ethanol production. Date extracts contain up to ~75% (w/w) sugars (mostly glucose, fructose, and sucrose), providing a rich carbon source for S. cerevisiae(Castillo et al., 2023). Prior studies confirm that S. cerevisiae can efficiently ferment date fruit substrates under the right conditions(Louhichi et al., 2013). Likewise, molasses – the thick syrup by-product of sugar cane or beet sugar extraction – is a well-established feedstock for industrial ethanol fermentation. Blackstrap molasses typically contains ~50% fermentable sugars along with minerals and trace nutrients. Its low cost and abundance (as a waste product of the sugar industry) make it an economically favorable substrate(Tesfaw & Assefa, 2014). However, both date juice and molasses share a critical challenge: nutrient imbalance. These substrates are rich in sugars but often deficient in key nutrients (particularly assimilable nitrogen) that yeast require for optimal growth and ethanol biosynthesis(Alminderej et al., 2022; Huertas-Díaz, 1990). As a result, fermentations on raw date juice or molasses can suffer from sluggish yeast growth, low ethanol yields, or stuck fermentation if not supplemented properly. This has motivated researchers to enrich such media with additional nutrients and to fine-tune environmental parameters to enhance fermentation performance. Among the environmental factors, pH of the fermentation medium is a crucial determinant of yeast activity and ethanol yield. S. cerevisiae thrives in mildly acidic conditions; if the medium is too acidic or too alkaline, yeast metabolic enzymes can be inactivated and fermentation rates decline(Phong et al., 2022). Studies have shown that while S. cerevisiae can grow across pH 4.0–6.0, ethanol production is optimal around pH 5.0–5.5. For example, Singh and Bishnoi found pH 5.5 to be the optimum for fermenting pretreated wheat straw, yielding significantly higher ethanol than at lower pH(Singh & Bishnoi, 2013). Similarly, Izmirlioglu and Demirci reported maximal ethanol output from potato waste at an initial pH of 5.5(Izmirlioglu & Demirci, 2012). Maintaining pH in this range favors yeast metabolic efficiency and also helps minimize bacterial contamination in high-sugar mashes. In high-solids fermentations (e.g. ≥30% dissolved sugars, as with concentrated molasses or date syrup), setting the mash pH to about 5.0–5.5 at the start is recommended to maximize ethanol yield and reduce the growth of undesired microbes(Narendranath & Power, 2005).

In contrast, uncontrolled fermentations can experience pH drops (especially when ammonium-based nutrients are used, which release acidity) leading to stressed yeast and suboptimal ethanol output(Yang et al., 2021). Therefore, regulating the initial pH to an optimal value and buffering the medium as needed are essential strategies to enhance fermentation efficiency on sugary substrates like date juice and molasses. Adequate nitrogen availability is another key factor for efficient ethanol fermentation. Yeast require assimilable nitrogen for protein synthesis, enzyme production, and biomass growth, all of which underpin robust fermentation(Shokri et al., 2021). Sugar-rich fruit juices and molasses often contain only limited yeast-assimilable nitrogen (YAN). For instance, blackstrap molasses may have only 0.5–1.5% nitrogen by weight(Huertas-Díaz, 1990), which can become limiting under high sugar (high C/N ratio) fermentation conditions. Nitrogen deficiency is known to slow yeast growth and prolong fermentation, as it can inhibit the synthesis of transport proteins needed for sugar uptake(Mirsalami & Alihosseini, 2023)). A variety of nutrient additives have been explored, including inorganic salts (e.g. ammonium sulfate or ammonium phosphate), organic nutrients (peptone, yeast extract), or urea(Duhan et al., 2013). Among these, urea ($CO(NH_2)_2$) has garnered attention as an inexpensive, high-nitrogen (46% N) source that is readily consumed by yeast(Yang et al., 2021). Urea supplementation can significantly boost ethanol productivity in high-sugar fermentations(Appiah-Nkansah et al., 2018; Thatiyamanee et al., 2025). In fact, in industrial fuel ethanol production, urea is widely used and often yields higher ethanol titers compared to equivalent ammonium salt

additions(Yang et al., 2021). For example, adding a moderate concentration of urea (on the order of a few grams per liter) to nitrogen-poor juices has been shown to accelerate fermentation and raise ethanol output, whereas unsupplemented batches might stall or leave residual sugars(Alihosseini et al., 2015). It should be noted that urea is avoided in beverage alcohol fermentations due to formation of ethyl carbamate, but for biofuel ethanol this is not a concern(Thanapornsin et al., 2025).

While date juice and molasses have each been investigated as substrates for ethanol production, systematic optimization of fermentation conditions tailored to these feedstocks remains limited. Many prior studies have examined either date extracts or molasses under preset or unoptimized conditions, often using default nutrient levels or uncontrolled pH (Lin et al., 2012). A significant gap exists in understanding the combined effects of pH and nitrogen supplementation—particularly urea, a cost-effective alternative to conventional nutrients—on fermentation performance. Moreover, date juice and molasses differ in sugar composition and inherent nutrients, suggesting that optimal strategies may be substrate-specific. Understanding how pH and urea concentration interact to influence *S. cerevisiae* fermentation is crucial for maximizing ethanol yield and productivity, addressing key bottlenecks such as low yields and prolonged fermentation times that limit economic viability (Alminderej et al., 2022).

Therefore, the aim of this study was to systematically optimize pH and urea concentration for bioethanol production from date juice and molasses using Saccharomyces cerevisiae. The specific objectives were to: (1) determine the optimal initial pH (3.5–5.5) for maximum ethanol production from both substrates, (2) evaluate the effect of urea supplementation (0.25–1.0% w/v) as a nitrogen source on fermentation efficiency, (3) compare the fermentation kinetics and ethanol yields between date juice and molasses under optimized conditions, and (4) identify practical fermentation parameters that can enhance the economic viability of bioethanol production from low-cost agricultural feedstocks. By addressing these objectives, this research provides a practical framework for converting abundant agricultural by-products into renewable biofuel, contributing to sustainable energy production and waste valorization.

2. Materials and Methods

2.1. Materials and Equipment

All chemicals used were of analytical grade. The microorganism *Saccharomyces cerevisiae* was obtained in commercial dry yeast form. Details of the chemicals and equipment are presented in Table 1. Industrial sugarcane molasses was obtained from a local sugar refinery in Khuzestan Province, Iran, and contained approximately 48–52% (w/w) fermentable sugars (mainly sucrose, glucose, and fructose) based on supplier specifications. Date juice (date syrup) was obtained from low-grade, unsalable Kabkab dates processed by a local supplier in Khuzestan Province. The date juice was filtered to remove suspended solids and diluted with distilled water to adjust the desired sugar concentration. No chemical pretreatment was applied to either substrate. For process standardization, both substrates were adjusted to 30% (w/v) total sugars and sterilized by autoclaving at 121°C for 15 min. After cooling, the sterilized substrate solutions were diluted with sterile distilled water to obtain a working fermentation medium with an initial sugar concentration of 10% (w/v) prior to inoculation.

2.2. Inoculum Preparation

For inoculum development, 100 mL of *S. cerevisiae* broth medium was prepared in a 500 mL Erlenmeyer flask and sterilized by autoclaving at 121°C for 15 minutes. After cooling to room temperature, the medium was aseptically inoculated with 2 g of dry yeast granules and incubated at 28°C in a shaking orbital incubator (150 rpm) for 48 hours to obtain an actively growing yeast culture.

Table 1: Chemicals and equipment used in the study

Category	Item	Specification / Model	Manufacturer / Supplier
Chemicals	Sodium hydroxide (NaOH)	Analytical grade	Merck, Germany
	Hydrochloric acid (HCl)	Analytical grade	Merck, Germany
	Urea	≥99% purity	Sigma-Aldrich, USA
	Glucose	D-(+)-Glucose, analytical grade	Merck, Germany
Microorganism	Saccharomyces cerevisiae	Commercial dry yeast	Instant Yeast, Iran
Equipment	Shaking orbital incubator	Temperature-controlled, 28°C ± 1°C	Memmert, Germany
	pH meter	Model SevenCompact™ S210	Mettler Toledo, Switzerland
	Spectrophotometer	UV-Vis model	Shimadzu, Japan
	Digital Alcohol Meter	ALM-155	ALM Instruments
	Autoclave	121°C, 15 psi	Systec, Germany

2.3. pH and Urea Optimization Experiments

To determine the optimal pH for ethanol production, five initial pH levels (3.5, 4.0, 4.5, 5.0, and 5.5) were evaluated. Although the substrates were initially prepared as 30% (w/v) stock solutions, the fermentation medium was diluted before inoculation, resulting in an effective initial sugar concentration of approximately 10% (w/v) during the fermentation process and the inoculum volume was set at 10 mL per 200 mL working volume. pH adjustment was performed using 1 M NaOH or 1 M HCl, and the final volume was brought to 200 mL with distilled water. Fermentation was carried out at 28°C in a shaking incubator (150 rpm) for 9 days. Ethanol concentration was determined by simple distillation followed by measurement with a digital alcohol meter and is reported as % (v/v). Briefly, 100 mL of fermented sample was distilled under atmospheric pressure, and ~50 mL of distillate was collected, cooled to 20°C, and measured using a digital alcohol meter (ALM-155; range 0–100% v/v; accuracy ±0.2% v/v). The instrument was calibrated prior to measurements using ethanol–water standards (5%, 10%, and 15% v/v) prepared from absolute ethanol (≥99.5%) and distilled water. All measurements were performed in triplicate. Four urea concentrations (0.25%, 0.5%, 0.75%, and 1.0% w/v) were tested under fixed conditions of 30% sugar concentration and pH 4.5. In each case, 10 mL of inoculum was added to a 200 mL working volume.

2.4. Final Fermentation under Optimized Conditions

The final ethanol production trial was conducted using the optimized parameters identified in Sections 2.3 and 2.4. Fermentation was maintained at 28°C for 9 days, after which ethanol yield was measured as described previously.

2.5. Statistical Analysis and Productivity calculation

All experiments were conducted in triplicate. Statistical analyses were performed using two-way ANOVA, with significance set at $p < 0.05$. Productivity was calculated based on the maximum ethanol concentration. Ethanol concentration was converted from % (v/v) to g/L using the density of ethanol at 20°C (0.789 g/mL) as shown in Eqs. (1)–(2):

$$C_{max}(g/L) = \%(v/v) \times 10 \times 0.789 = \%(v/v) \times 7.89 \quad (1)$$

$$\text{Productivity (g/L/day)} = \frac{C_{max}(g/L)}{t_{max}(day)} \quad (2)$$

where $C_{max}$is the maximum ethanol concentration and $t_{max}$is the fermentation time (days) at which $C_{max}$was reached.

3.Results

Initial pH significantly influenced ethanol production in both substrates (Figure 1, Table 2). The optimal pH for maximum ethanol yield was 4.5 for both molasses and date juice, followed closely by pH 4.0. Deviations from this optimum, particularly at extreme pH values (3.5 and 5.5), resulted in substantially lower ethanol concentrations. Across all pH treatments, ethanol production increased rapidly during the first 5–6 days before plateauing between days 7 and 9, indicating completion of fermentation. These findings demonstrate that pH 4.5 provides the most favorable conditions for S. cerevisiae fermentation on both date juice and molasses substrates.

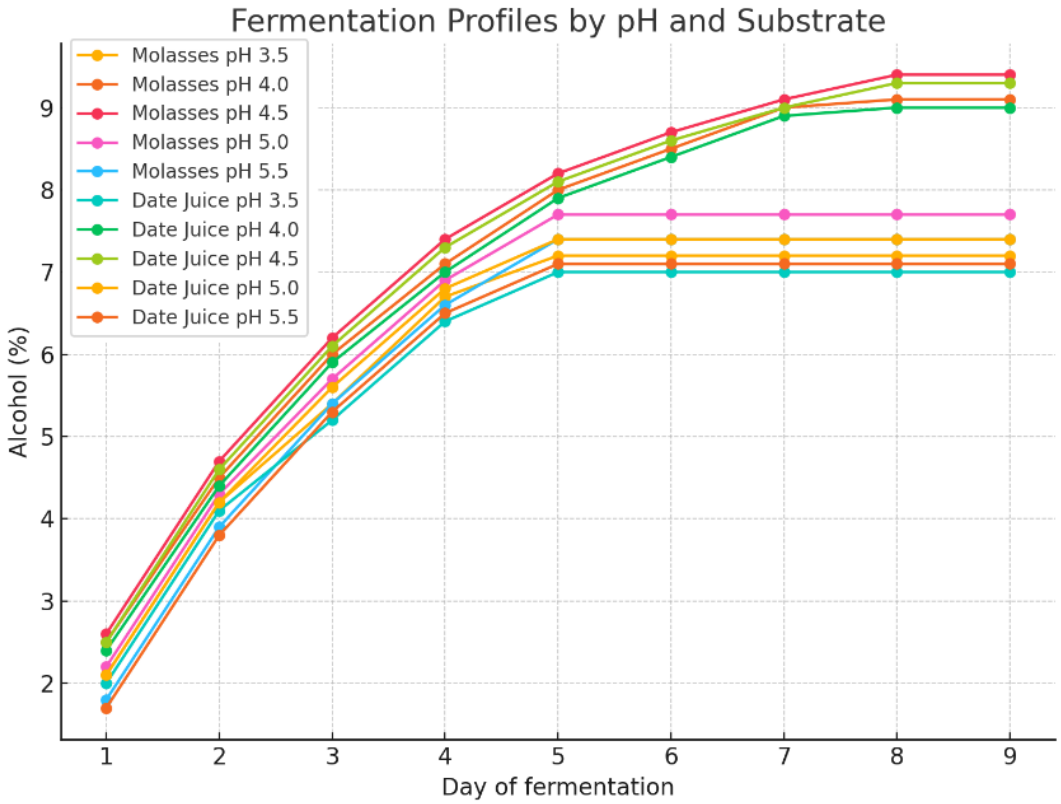

Figure 1. Ethanol concentration at different initial pH levels and substrate types

Table 2 summarizes the maximum ethanol concentration, time to reach maximum (t_max), and productivity for both substrates across five pH levels. pH 4.5 yielded the highest ethanol concentrations in both molasses and date juice, followed by pH 4.0, while extreme pH values resulted in lower yields. Notably, fermentations at pH 5.0 and 5.5 exhibited higher productivity despite lower final ethanol concentrations, suggesting faster but less complete sugar conversion. These results confirm pH 4.5 as optimal for maximizing ethanol yield, whereas pH 5.5 may favor faster fermentation rates.

Table 2. Maximum ethanol concentration, time to reach maximum

Substrate	pH	max_alcohol (% v/v)	t_max (day)	C_max (g/L)	Productivity (g/L/day)
	3.5	7.2	5	56.81	11.36
	4	9.1	8	71.8	8.98
Molasses	4.5	9.4	8	74.17	9.27
	5	7.7	5	60.75	12.15
	5.5	7.4	5	58.39	11.68
	3.5	7	5	55.23	11.05
	4	9	8	71.01	8.88
Date Juice	4.5	9.3	8	73.38	9.17
	5	7.4	5	58.39	11.68
	5.5	7.1	5	56.02	11.2

Note: max_alcohol = maximum ethanol concentration (% v/v); t_max = time to reach maximum ethanol concentration (days).

Urea concentration significantly influenced ethanol production in both substrates (Figure 2, Table 3). The optimal urea level was 0.5% (w/v) for both molasses and date juice, yielding the highest ethanol concentrations. Lower urea supplementation (0.25%) resulted in moderately reduced yields, while higher concentrations (0.75% and 1.0%) led to substantially lower ethanol production, suggesting that excess nitrogen inhibits fermentation. Similar to pH experiments, ethanol production increased rapidly during the first 5–6 days before plateauing between days 7 and 9, indicating fermentation completion.

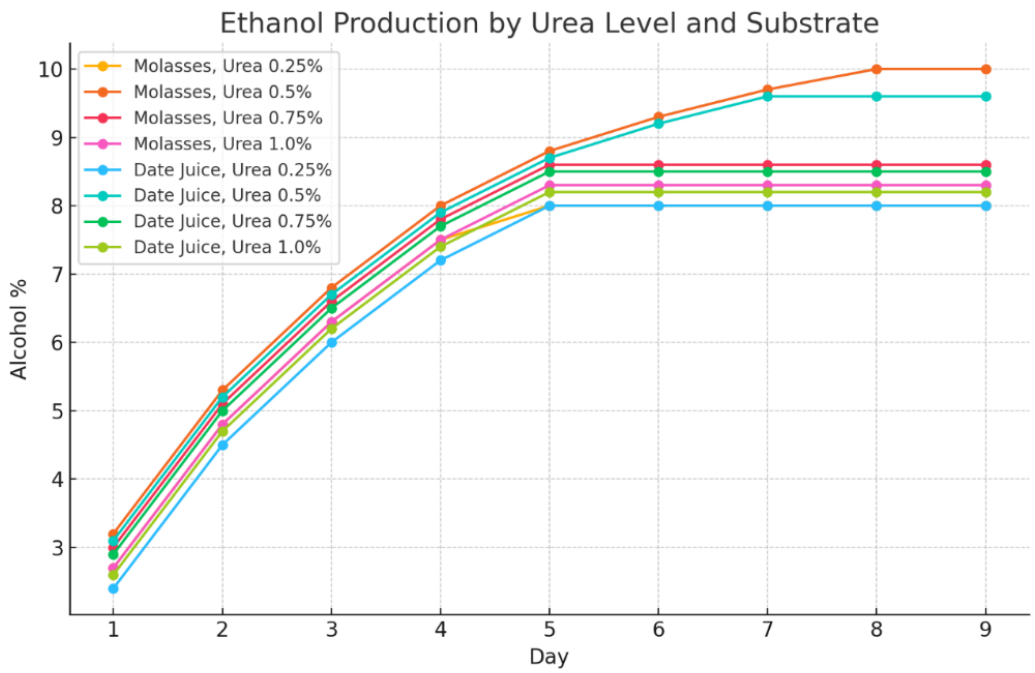

Figure 2: Ethanol production profiles at different urea concentrations for molasses and date juice

Table 3 summarizes the maximum ethanol concentration, time to reach maximum (t_max), and productivity at different urea levels. As observed with pH optimization, 0.5% urea yielded the highest ethanol concentrations for both substrates, although other urea levels exhibited higher productivity due to earlier fermentation completion.

Table 3. Maximum ethanol yield, t_max, and productivity at different urea concentrations

Substrate	Urea (% w/v)	max_alcohol (% v/v)	t_max (day)	C_max (g/L)	Productivity (g/L/day)
Molasses	0.25	8	5	63.12	12.62
	0.5	10	8	78.9	9.86
	0.75	8.6	5	67.85	13.57
	1	8.3	5	65.49	13.1
Date Juice	0.25	8	5	63.12	12.62
	0.5	9.6	7	75.74	10.82
	0.75	8.5	5	67.06	13.41
	1	8.2	5	64.7	12.94

Note: max_alcohol = maximum ethanol concentration (% v/v); t_max = time to reach maximum ethanol concentration (days).

Figure 3 compares ethanol production from molasses and date juice at the optimal pH of 4.5. Both substrates exhibited similar fermentation kinetics, with rapid ethanol accumulation during the first five days followed by stabilization between days 7 and 9. Molasses achieved marginally higher final ethanol yields than date juice, confirming efficient fermentation performance under optimized pH conditions.

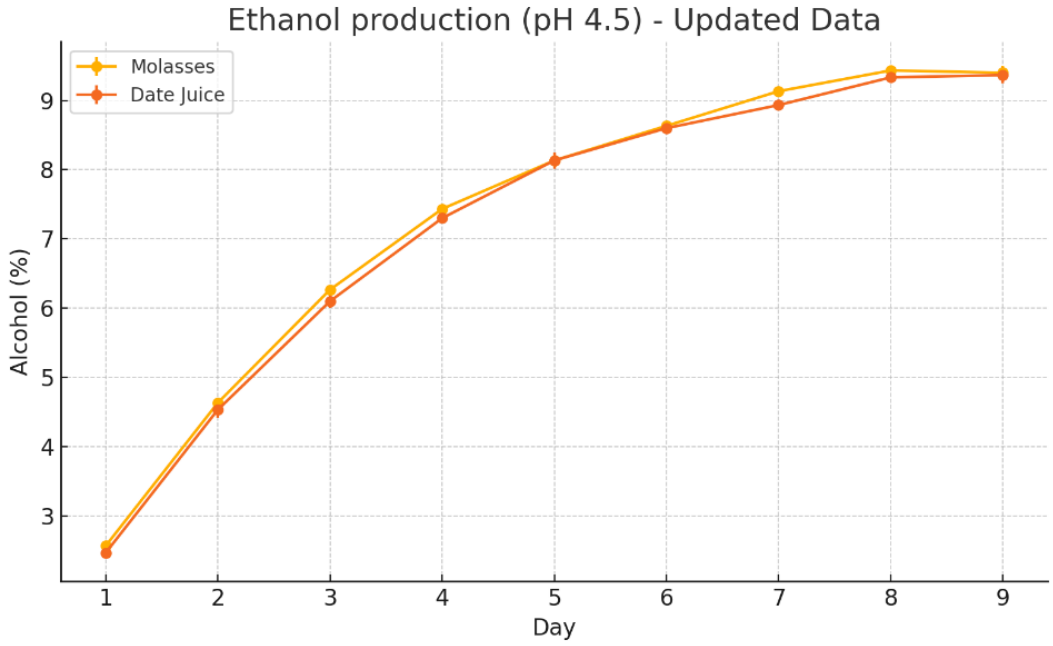

Figure 3. Ethanol production at pH 4.5 for molasses and date juice substrates over a 9-day fermentation period

Similarly, Figure 4 compares ethanol production at the optimal urea concentration of 0.5% (w/v). Both substrates displayed comparable fermentation kinetics, with rapid ethanol accumulation followed by stabilization. Molasses

again produced slightly higher final ethanol yields than date juice, confirming 0.5% urea as the optimal nitrogen supplementation level for both substrates.

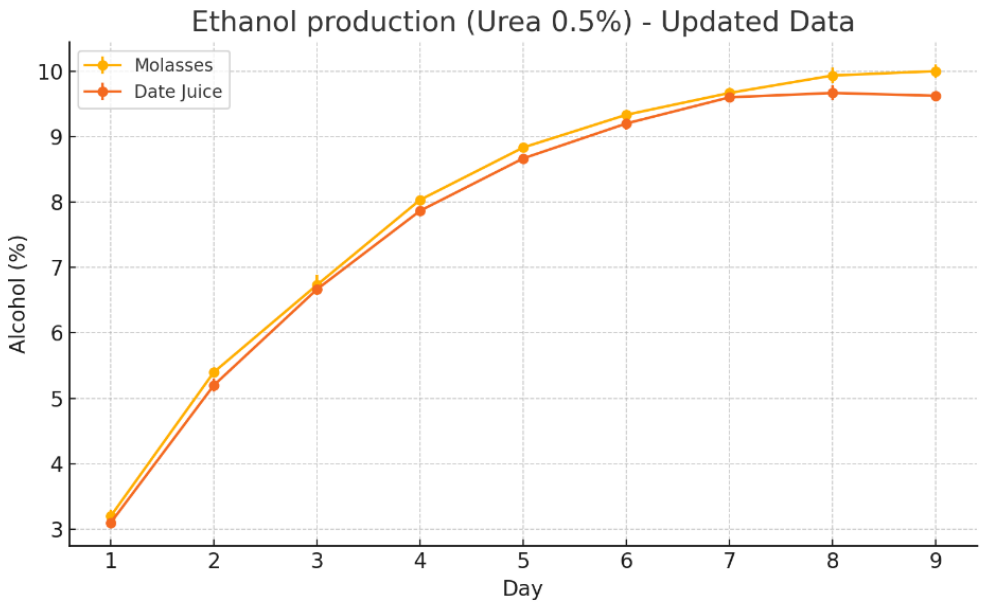

Figure 4. Ethanol production at 0.5% urea for molasses and date juice substrates over a 9-day fermentation period

Two-way ANOVA revealed significant effects of both substrate type and fermentation day on ethanol production under pH and urea optimization conditions (Table 4). For pH experiments, both substrate ($p < 0.001$) and day ($p < 0.001$) had significant main effects on ethanol concentration, but their interaction was not significant ($p = 0.395$), indicating that both molasses and date juice followed similar fermentation kinetics across all pH levels. Similarly, under urea optimization, substrate ($p < 0.001$) and day ($p < 0.001$) showed highly significant main effects. However, a significant substrate × day interaction was also observed ($p = 0.037$), suggesting that the temporal response to urea supplementation differed slightly between the two substrates.

Table 4. Two-way ANOVA results under different pH and urea conditions

Condition	Source	Sum of Squares	df	F	p-value
pH	Substrate	0.1252	1	21.125	5.12×10^{-5}
	Day	273.5833	8	5770.898	1.49×10^{-53}
	Substrate × Day	0.0515	8	1.086	0.395
	Residual	0.2133	36	—	—
Urea	Substrate	0.397	1	62.154	2.36×10^{-9}
	Day	261.3817	8	5115.473	1.30×10^{-52}
	Substrate × Day	0.1208	8	2.365	0.037
	Residual	0.2299	36	—	—

4. Discussion

This study systematically optimized pH and urea concentration for bioethanol production from date juice and molasses using S. cerevisiae. The results demonstrate that both environmental factors significantly influence fermentation efficiency, with pH 4.5 and 0.5% urea yielding maximum ethanol concentrations for both substrates. The optimal pH of 4.5 observed in this study aligns with previous findings on yeast fermentation. Singh and Bishnoi (2013) reported pH 5.5 as optimal for wheat straw, while Izmirlioglu and Demirci (2012) found pH 5.5 ideal for potato waste. The slightly lower optimum in our work may reflect the specific composition of molasses and date juice, which contain organic acids that influence buffering capacity. At extreme pH values (3.5 and 5.5), ethanol yields decreased substantially, consistent with the known sensitivity of glycolytic enzymes to pH stress. Acidic conditions likely inhibit hexose transporters and fermentative enzymes, while alkaline pH may promote undesirable microbial growth and reduce yeast metabolic efficiency (Narendranath and Power, 2005). The absence of a significant substrate × day interaction under pH optimization indicates that both substrates respond similarly to pH manipulation, suggesting that optimization strategies can be reliably applied to either feedstock.

Urea supplementation at 0.5% (w/v) enhanced ethanol production in both substrates, supporting previous reports that nitrogen availability limits fermentation of sugar-rich but nitrogen-poor feedstocks (Yang et al., 2021). At this concentration, urea provides sufficient assimilable nitrogen for yeast growth and enzyme synthesis without causing osmotic or metabolic stress. The inhibitory effect of higher urea concentrations (0.75% and 1.0%) may result from ammonia toxicity or osmotic imbalance, as excess nitrogen can disrupt cellular ion homeostasis (Rojo et al., 2023)(Rojo et al., 2023). The significant substrate × day interaction under urea optimization suggests substrate-specific differences in nitrogen utilization kinetics, highlighting the importance of tailoring nitrogen supplementation to substrate composition.

Both substrates achieved comparable maximum ethanol yields (~9–10% v/v), confirming their suitability for bioethanol production. Molasses consistently produced slightly higher yields than date juice, likely due to differences in trace element composition. The optimized conditions identified here (pH 4.5, 0.5% urea) provide a practical framework for industrial-scale bioethanol production from low-cost feedstocks. Urea is significantly cheaper than organic nitrogen sources, making this approach economically attractive. The high ethanol yields achieved are comparable to or exceed those reported in similar studies(Louhichi et al., 2013; Tesfaw & Assefa, 2014). Future research should investigate continuous fermentation systems and thermotolerant yeast strains to further enhance process economics.

5. Conclusion

This study successfully identified optimal fermentation conditions for bioethanol production from date juice and molasses, two abundant agricultural by-products. Both pH and urea supplementation significantly influenced fermentation efficiency, with pH 4.5 and 0.5% (w/v) urea providing the best performance for both substrates. The comparable ethanol yields achieved from date juice and molasses demonstrate the versatility of *Saccharomyces cerevisiae* in fermenting diverse sugar-rich feedstocks under optimized conditions. These findings have important implications for sustainable biofuel production and agricultural waste valorization. By converting low-value by-products into renewable energy, this approach addresses both waste management challenges and the need for fossil fuel alternatives. The use of inexpensive urea as a nitrogen source further enhances economic feasibility, making this strategy particularly attractive for developing regions with abundant date palm cultivation and sugarcane processing industries. The optimization framework developed here can be adapted to other agricultural substrates, supporting circular economy principles and contributing to rural economic development through decentralized biofuel production.

limitations statement

It should be acknowledged that the experiments in this study were conducted at laboratory scale. Further validation is needed for industrial or field-scale application to confirm the robustness and economic feasibility of the optimized fermentation conditions under commercial production settings.

Declaration

Acknowledgements: We are grateful to all participants for their contributions to the present study.

Funding: Not applicable

Conflict of Interest Disclosures: The authors declare that they have no known financial or personal relationships that could have appeared to influence the work reported in this paper. There are no competing interests, either commercial or non-commercial, associated with this study.

Ethics approval/declaration: Not applicable

Consent to participate: Not applicable

Consent for publication: Not applicable

Data availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Authors' contribution: A. Ali Hosseini: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft. H. Nikokar: Data curation, Resources, writing – original draft. R.Nasirian : Writing – original draft, Project administration, Investigation, Resource

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How to cite this article:

Nikokar, H., Robabeh Nasirian, & Alihosseini , A. (2026). Sustainable Utilization of Agricultural Sugar Substrates for Bioethanol Production. Journal of Agriculture Sustainability and Environment, 5(1). https://doi.org/10.56556/jase.v5i1.1413