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Evaluating Kernza (*Thinopyrum intermedium*) as a Co-Fermentation Feedstock for Bioethanol Production

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ABSTRACT

Background: Kernza (*Thinopyrum intermedium*), a perennial grain developed by the Land Institute, presents a promising alternative to conventional ethanol feedstocks due to its deep-root systems, low fertilizer requirements, and carbon sequestration potential.

Objectives: This study evaluates the fermentation performance of Kernza, alone and in combination with corn, under two temperature conditions (25°C and 30°C). Five substrate ratios were tested using *Saccharomyces cerevisiae*: 100% corn, 75%:25% corn-Kernza, 50:50, 25:75, and 100% Kernza.

Methods: Ethanol yield was estimated via CO_2 weight loss corrected for evaporation. Carbohydrate availability was derived from literature values. Statistical analysis examined the effects of substrate composition and temperature on ethanol production.

Results: The 75:25 corn-Kernza blend at 25°C produced the highest ethanol yield, suggesting synergistic interactions. Conversely, higher Kernza proportions reduced yield, likely due to its higher fiber and low starch content. pH monitoring indicated that Kernza inclusion influenced medium buffering.

Conclusion: Kernza is a viable supplemental feedstock when used in moderate proportions with corn. Further optimization, including enzymatic pretreatment, may enhance its fermentation potential.

1 | Introduction

The global pursuit of renewable energy has intensified research into sustainable biofuel sources that balance environmental benefits with fermentation efficiency. In the United States, corn remains the dominant feedstock for ethanol production due to its high starch content and well-characterized fermentation profile (Renewable Fuels Association 2025). However, concerns over resource-intensive monocultures have prompted interest in alternative, low-input crops (Tilman et al. 2009).

Many approaches to sustainable or regenerative agriculture have seen various levels of success in research trials and then adoption by farmers. Kernza (*Thinopyrum intermedium*), a perennial grain developed by the Land Institute (https://landinstitute.org), offers several agronomic and ecological advantages over annual cereals. Its extensive root system promotes soil carbon sequestration, reduces nitrate leaching, and decreases dependence on fertilizers (Kantar et al. 2016). Thorough reviews of its agronomic characteristics are found elsewhere. Despite these environmental benefits, Kernza applications in food, feed, and industrial applications are lacking. This is especially true in industrial fermentation systems, such as bioethanol production, where it currently remains unexplored. Its high fiber and arabinoxylan content may pose challenges for sugar accessibility, particularly in the absence of enzymatic pretreatment. (Goldbeck et al. 2014). Prior research on high-fiber feedstocks, such as wheat bran and barley straw, has demonstrated the importance of tailored enzymatic strategies to overcome hemicellulose barriers. Building on this foundation,

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the present study investigates the fermentation potential of Kernza in unhydrolyzed form both as a standalone substrate and in blends with corn.

The main objective of this research study was to examine whether moderate Kernza inclusion could enhance ethanol production efficiency when combined with corn, leveraging Kernza's micronutrient profile while benefiting from corn's fermentable starch content. This study explored the interactive effects of substrate composition and temperature on ethanol yield, supported by pH monitoring and statistical modeling to identify optimal conditions for future bioethanol integrations.

2 | Materials and Methods

2.1 | Substrate Selection and Preparation

Two primary substrates were used: yellow dent corn (*Zea mays*) and Kernza (*T. intermedium*), a perennial intermediate wheatgrass grain developed by the Land Institute (Craine and DeHaan 2024). Corn was selected for its high starch content, for its potential as a sustainable feedstock with a distinctive fiber profile.

Moisture content was measured using a GAC-UGMA grain moisture meter. Corn samples contained 12.9% moisture, and Kernza 10.9%. Both grains were milled using a Glen Mills hammermill equipped with a 1 mm screen. The resulting flour was sieved using a W.S. Tyler Roto Tap shaker to isolate particles $< 180\,\mu m$, a size chosen to enhance fermentability through improved yeast–substrate interaction.

2.1.1 | *Fermentation Medium and Inoculation*

Fermentation was conducted using *Saccharomyces cerevisiae* (Red Star DADY), a distiller's yeast commonly used in ethanol production due to its robust fermentation profile in high-starch environments (Wang et al. 2009).

Each fermentation was conducted in 250 mL Erlenmeyer flasks that contained 1 g of Red DADY, 2 g of dry substrate (corn, Kernza, or blend), and 100 mL of distilled water. The blends consisted of five substrate conditions: 100% corn, 75:25 corn:-Kernza, 50:50 corn:Kernza, 25:75 corn:Kernza, and 100 Kernza. The inclusion of a control flask (uninoculated) was prepared to quantify evaporative losses.

2.2 | Sterilization and Incubation Conditions

All fermentation flasks were sterilized by autoclaving at 121 C for 60 min. After cooling to room temperature (approximately 25°C), flasks were inoculated with *S. cerevisiae*. Water-lock traps were fitted to maintain sterility and allow CO_2 release.

Fermentations were carried out over 144 h (6 days) in a New Brunswick Excella E24 incubator shaker set at 110 rpm. Two temperature conditions were tested: 25°C and 30°C. Each treatment group was replicated in triplicate ($n = 3$) for statistical robustness.

2.2.1 | *Ethanol Yield Estimation*

Flask weights were recorded daily using an OHAUS Explorer precision balance. CO_2 loss (as an indirect indicator of ethanol production) was calculated as:

$$\text{Ethanol Yield (\%)} = 100 \times \frac{46 \times (\text{mass lost in grams})}{44 \times (\text{mass of carbohydrates})},$$

TABLE 1 | All the fermentation groups with their respective crop blend and designated temperature for experimental setup and tracking of measurements for each one.

Experiment number	Blend	Temperature
1	JW	30°C
2	JW	30°C
3	JW	30°C
4	100% Corn	30°C
5	100% Corn	30°C
6	100% Corn	30°C
7	75:25% Corn-Kernza	30°C
8	75:25% Corn-Kernza	30°C
9	75:25% Corn-Kernza	30°C
10	50:50% Corn-Kernza	30°C
11	50:50% Corn-Kernza	30°C
12	50:50% Corn-Kernza	30°C
13	25:75% Corn-Kernza	30°C
14	25:75% Corn-Kernza	30°C
15	25:75% Corn-Kernza	30°C
16	100% Kernza	30°C
17	100% Kernza	30°C
18	100% Kernza	30°C
19	JW	25°C
20	JW	25°C
21	JW	25°C
22	100% Corn	25°C
23	100% Corn	25°C
24	100% Corn	25°C
25	75:25% Corn-Kernza	25°C
26	75:25% Corn-Kernza	25°C
27	75:25% Corn-Kernza	25°C
28	50:50% Corn-Kernza	25°C
29	50:50% Corn-Kernza	25°C
30	50:50% Corn-Kernza	25°C
31	25:75% Corn-Kernza	25°C
32	25:75% Corn-Kernza	25°C
33	25:75% Corn-Kernza	25°C
34	100% Kernza	25°C
35	100% Kernza	25°C
36	100% Kernza	25°C

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where 46 g/mol is the molecular weight of ethanol and 44 g/mol is that of CO_2. Evaporative losses were subtracted using control flask values.

2.2.2 | *pH Monitoring*

pH was measured daily using a Mettler Toledo FiveEasy Plus pH meter, calibrated with standard 4.0 and 7.0 buffers. pH trends were used to monitor yeast metabolic activity and assess the buffering capacity of each substrate.

2.3 | Carbohydrate Quantification

The carbohydrate content for each substrate was obtained from published literature values for corn (Corn Grain, Yellow—Nutrients—SR Legacy|USDA FoodData Central 2019) and Kernza (Craine and DeHaan 2024). Table 6 presents the calculated carbohydrate load per 200 mL for each substrate ratio.

TABLE 2 | Summary of fit describes specific parameters that help understand the model's validity and how well the data are represented in the study.

Summary of fit	Value
R^2	0.959318
Adjusted R^2	0.955443
Root mean square error	6.372142
Mean of response	41.79953
Observations (sum of weights)	70

Note: An R^2 of 96% proves that the model used, this being ANOVA, could provide values close to the expected results. An adjusted R^2 of 95% signifies the small error the model achieved; this gives the results another level of veracity.

3 | Statistical Analysis

3.1 | Data Analysis Setup

The statistical analysis was done using JMP 17 Pro to evaluate the effects of our independent variables (substrate concentration and incubation temperatures, Table 1) and their interaction with the ethanol yield produced in a 6-day fermentation. The time variable was considered a continuous variable to show the progression of fermentation every 24 h.

3.2 | Statistical Methods

In the methods, several tests helped predict whether the data was relevant. One aspect of this analysis involved evaluating the fit of the model by using the coefficient of determination (R^2), which quantifies how well the model explains the variability in the observed data. At the same time, the adjusted R^2 relates to the adjusted number of predictors in the model. The fitting test also used root mean square error (RMSE) to measure the average prediction error. Finally, the model fit test showed that the residual plot examined the normality and homogeneity of the data, including the variance. The alpha level for all the analyses was 0.05.

Regarding analysis of variance (ANOVA), a two-way form was used to evaluate the main effects, including the substrate composition, incubation temperature, and the interactions between both. The methods also included post hoc analysis like Tukey's Honest Significant Difference (HSD), which is used to identify pairwise differences between the groups. In the effects test, the F test was used to determine the significance of each group of independent variables.

Equations were provided for further discussion of and validity of methods. Equation (1) shows how the RMSE is calculated and represents a critical step in understanding the model's accuracy and how well it fits together.

TABLE 3 | The ANOVA table describes the model's characteristics and how the averages compare to the assessed analysis.

Source	*df*	Sum of squares	Mean square	*F* ratio	Prob > *F*
Model	6	60,320.948	10,053.5	247.5974	
Error	63	2558.064	40.6		< 0.0001*
C. Total	69	62,879.012			

Note: The sum of squares, being an extremely high value, dictates the correlation in the model and the accuracy of the said model; the *F* ratio symbolizes how strongly the model fits in the ANOVA model, predicting that this was the best choice to see if the variables put to the test would be reliable statistics. * Indicate significance level of 0.05.

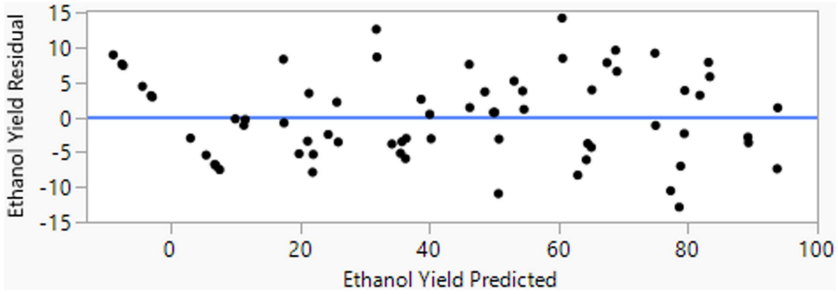


FIGURE 1 | Residuals plotted against predicted ethanol yield across all treatments. The lack of a discernible pattern supports model assumptions of homoscedasticity and normal distribution of errors. [Color figure can be viewed at wileyonlinelibrary.com]

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$$\text{RMSE} = \sqrt{\frac{\sum_{i=0}^{n}(y_i - \widehat{y})^2}{n}}, \quad (1)$$

where, y_i = observed values of the dependent variables; y = predicted values from the model; n = total number of observations.

The *F*-test, seen in the effect test table, reflects the importance of understanding the significance between the groups and what influences the fermentation process the most to identify which groups are more impactful than others. These are explained from Equation (2) to (6):

$$F = \frac{\text{MS}_{\text{Between}}}{\text{MS}_{\text{Within}}}, \quad (2)$$

where, $\text{MS}_{\text{Between}}$ = mean square for between-group variability.

$$\text{MS}_{\text{Between}} = \frac{\text{SS}_{\text{Between}}}{df_{\text{Between}}}, \quad (3)$$

where, $\text{SS}_{\text{Between}}$ = sum of squares between groups; df_{Between} = degrees of freedom between groups, which is calculated as:

$$df_{\text{Between}} = \text{Number of Groups} - 1. \quad (4)$$

Then, we proceed to calculate the mean square within the group variability:

$$\text{MS}_{\text{Within}} = \frac{\text{SS}_{\text{Within}}}{df_{\text{Within}}}, \quad (5)$$

where, $\text{SS}_{\text{Within}}$ = sum of squares within groups (also referred to as the error or residual sum of squares); df_{Within} = Degrees of freedom Within Groups, that is calculated as:

$$df_{\text{Within}} = \text{Total Number of Observations} - \text{Number of Groups}. \quad (6)$$

The last concept discussed in the analysis is Tukey's HSD, as detailed in Equation (7).

The formula is as follows:

$$q = \frac{|\bar{X}_i - \bar{X}_j|}{\sqrt{\frac{\text{MS}_{\text{Within}}}{n}}}, \quad (7)$$

where X_i and X_j refer to the means of two groups that are being compared; $\text{MS}_{\text{Within}}$ = mean square within the groups (this number comes from the ANOVA, which represents the pooled within-group variance); n = number of observations in a group;

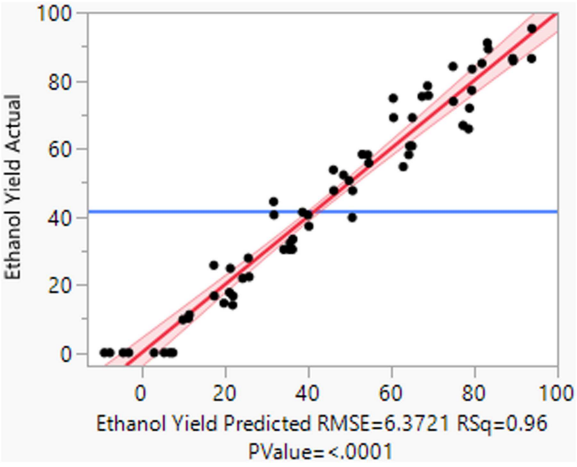


FIGURE 2 | Observed ethanol yield values versus predicted values from the ANOVA model. A strong linear relationship ($R^2 = 0.96$) indicates high model accuracy and low prediction error (RMSE = 6.37). [Color figure can be viewed at wileyonlinelibrary.com]

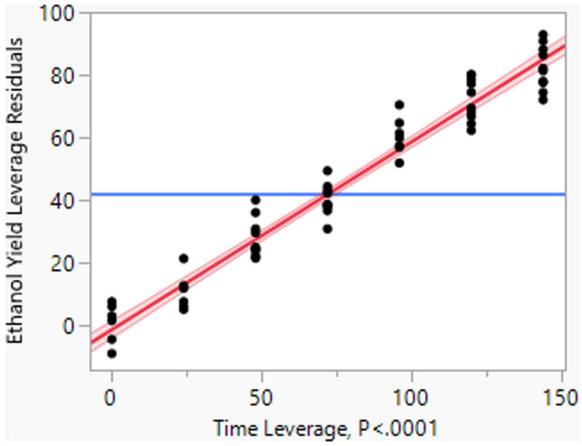


FIGURE 3 | Leverage plot for fermentation time as a predictor of ethanol yield. The steep regression line and significance ($p < 0.0001$) indicate that time was the most influential variable in the model. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 4 | The parameter estimates table reflects the different temperature and fermentation groups alongside time and intercept.

Term	Estimate	Standard error	*t* Ratio	Prob > |*t*|
Time	0.5991335	0.015867	37.76	< 0.0001*
Temperature group [25]	2.2611217	0.761617	2.97	0.0042*
Intercept	−1.338078	1.373024	−0.97	0.3335
Fermentation group [100 C]	6.5826041	1.523233	32	< 0.001*
Fermentation group [75_25 CK]	6.6743719	1.523233	38	< 0.001*
Fermentation group [50_50 CK]	−5.372544	1.523233	−3.53	0.0008*
Fermentation group [25_75 CK]	−3.838027	1.523233	−2.52	0.0143*

Note: It puts them against the values seen in the 30°C temperatures and groups, allowing us to see the most influential groups in the 25°C series. The fermentation group with the most significant change is the blend of 75:25% corn-Kernza, with a *p*-value < 0.001, meaning that including Kernza significantly affects ethanol production. Although the most influential, all the groups in this temperature also presented significant results. * Indicates significance level of 0.05.

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TABLE 5 | The effects test table presents the parameters from different sources and shows that each one has an influence on ethanol production.

Source	Nparm	*df*	Sum of squares	*F* ratio	Prob > *F*
Time	1	1	57,893.217	1425.794	< 0.0001*
Fermentation group	4	4	2069.844	12.7440	< 0.0001*
Temperature group	1	1	357.887	8.8140	0.0042*

Note: Time has an *F* ratio of 1425.794, which relates to how important time is to reflect higher ethanol yield—followed by fermentation with an *F* ratio of 12.7440. Even though it is not as high as time, it still presents itself as a fascinating finding, considering that the only ingredients in the blend are corn, distilled water, and Kernza, thus reinforcing the idea while also challenging the hypothesis of what is expected in a corn fermentation and what parameters dictate the success of a fermentation run. Moreover, even though it is in last place, temperature with an *F* value of 8.8140 also plays a significant role in ensuring that the enzymatic activity can be seen from two different temperatures, which challenges the idea that higher temperatures result in higher ethanol production. * Indicate significance level of 0.05.

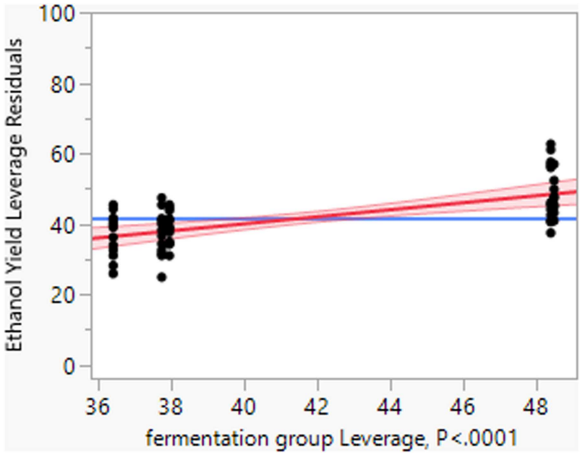


FIGURE 4 | Leverage plot for substrate composition (fermentation group). Substrate blend had a significant effect on ethanol yield ($p < 0.0001$), with the 75:25 corn-Kernza blend showing the strongest response. [Color figure can be viewed at wileyonlinelibrary.com]

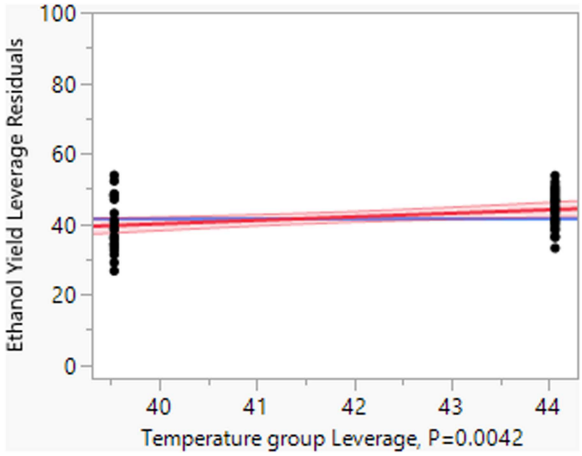


FIGURE 5 | Leverage plot for incubation temperature. Although temperature was statistically significant ($p = 0.0042$), its contribution to ethanol yield was less pronounced than other predictors. [Color figure can be viewed at wileyonlinelibrary.com]

q = the Tukey q-statistic, which follows the studentized range distribution.

4 | Results

4.1 | Model Performance and Statistical Significance

The statistical model demonstrated a strong fit with an R^2 of 0.959 and adjusted R^2 of 0.955 (Table 2), indicating that 95.5% of the variance in ethanol yield was explained by the independent variables. The overall model was significant ($F = 247.60$, $p < 0.0001$; Table 3), confirming the combined effects of substrate composition, fermentation temperature, and time on ethanol yield.

Residual analysis (Figure 1) showed no discernible pattern, supporting the assumption of homoscedasticity. The actual vs predicted values (Figure 2) confirmed high predictive accuracy, with an RMSE of 6.37 and no outlier behavior across treatment groups.

4.2 | Influence of Independent Variables

Table 4 summarizes the parameter estimates for each independent variable. Time exerted the most significant effect (Figure 3, $F = 1425.79$, $p < 0.0001$), followed by substrate composition ($F = 12.74$, $p < 0.0001$) and temperature ($F = 8.14$, $p = 0.0042$; Table 5). All three predictors were statistically significant.

Post hoc comparisons using Tukey's HSD revealed that the 75:25 corn-Kernza blend at 25°C yielded significantly more ethanol than all other treatment groups ($p < 0.00001$), while the 100% Kernza group consistently produced the lowest yield.

4.3 | Effect of Substrate Composition on Ethanol Yield

Substrate composition significantly influenced ethanol productivity (Table 5; Figure 4). The 100% corn group exhibited the highest carbohydrate content (1.492 g/200 mL), while 100% Kernza contained the lowest (1.378 g/200 mL; Table 6). Blends with increasing Kernza proportions showed a linear decrease in fermentable carbohydrate concentration.

Despite this, moderate Kernza inclusion (25%) enhanced ethanol yield beyond that of 100% corn. This synergistic effect may stem from micronutrients in Kernza that support yeast metabolism, particularly when not present in excess.

4.4 | Effect of Temperature and pH Trends

Fermentation at 25°C consistently outperformed 30°C across all substrate compositions ($F = 8.14$, $p = 0.0042$; Figure 5). Contrary to expectations based on yeast thermotolerance, elevated

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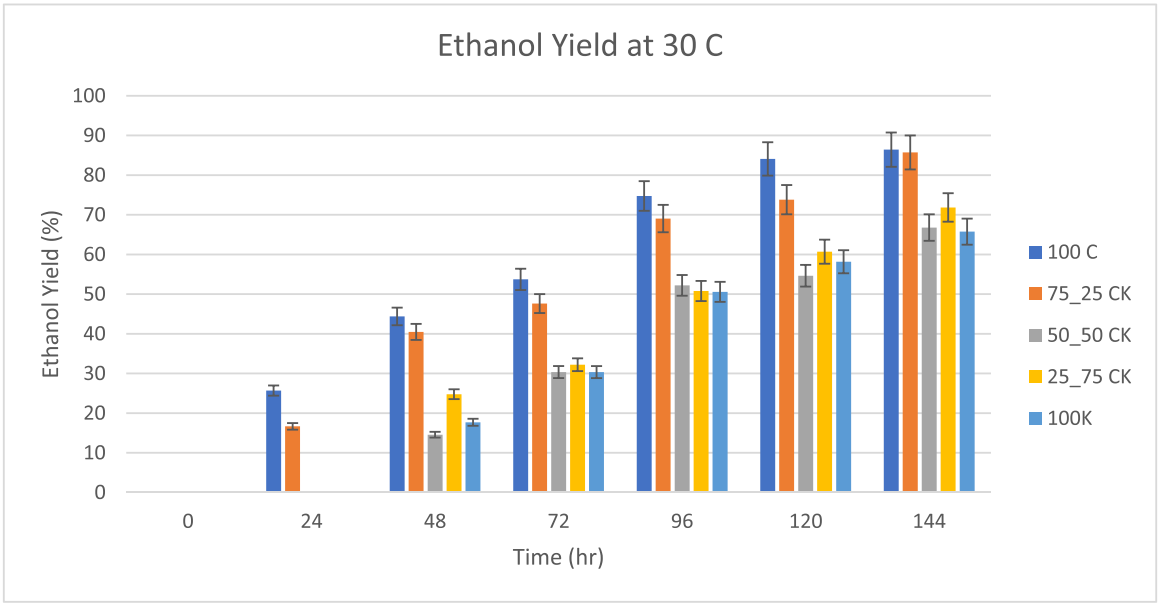


FIGURE 6 | Ethanol yield progression (+/− one standard deviation) over 144 h at 30°C for five substrate blends. Yields increased across all groups but plateaued earlier than 25°C. The 100% corn achieved the highest yield under these conditions. [Color figure can be viewed at wileyonlinelibrary.com]

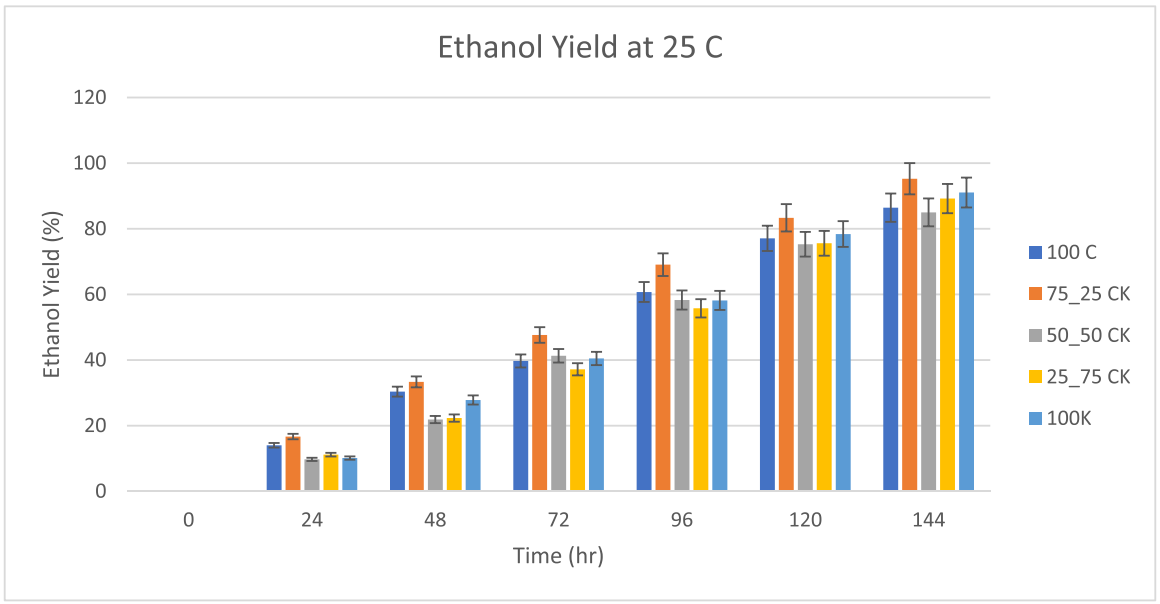


FIGURE 7 | Ethanol yield progression (+/− one standard deviation) over 144 h at 25°C for five substrate blends. The 75:25 corn-Kernza blend produced the highest ethanol yield, with a consistent upward trend and peak efficiency above 95% by 144 h. [Color figure can be viewed at wileyonlinelibrary.com]

temperatures led to reduced yield, potentially due to heat stress and accelerated by-product formation.

Figures 6 and 7 illustrate ethanol progression over 144 h for each temperature condition. At 25°C, the 75:25 blend surpassed 95% ethanol yield efficiency, while yields plateaued at lower levels under 30°C conditions.

pH monitoring indicated significant acidification during fermentation (Table 7). The largest pH drop occurred in the 100% Kernza group at 30°C (Δ pH = −2.34), suggesting higher metabolic acid production. At 25°C, pH changes were more stable, with less drastic shifts across all blends. The 75:25 blend again showed optimal behavior, with a relatively moderate pH decline (Δ pH = −1.40).

5 | Discussion

This study evaluated the fermentation potential of Kernza (*T. intermedium*), both independently and in combination with corn, under two incubation temperatures. The results indicate that substrate composition, temperature, and fermentation time each significantly affected ethanol yield.

The highest ethanol production was achieved using a 75:25 corn-Kernza blend fermented at 25°C. This suggests that moderate Kernza inclusion may offer synergistic benefits when combined with a high-starch substrate like corn. While Kernza alone resulted in lower yields due to its limited starch content and higher fiber concentration, its partial inclusion appeared to support yeast metabolism, potentially through trace nutrients or buffering effects.

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These findings align with previous studies demonstrating that blending high-starch and high-fiber feedstocks can improve fermentation outcomes (Yang et al. 2015). In particular, arabinoxylans and hemicelluloses in Kernza, when present in limited quantities, may provide co-factors or influence pH stability that support more efficient ethanol production(Vacilotto et al. 2022; Goldbeck et al. 2014).

Contrary to initial expectations, fermentation at 30°C did not enhance ethanol yield. Although the *S. cerevisiae* strain is often associated with high-temperature tolerance, performance declined at elevated temperatures across all substrate groups. This may be attributed to thermal stress impairing enzymatic function and promoting the formation of unwanted by-products, as suggested in prior work on temperature-sensitive fermentation pathways.

pH monitoring further revealed that Kernza-containing blends exhibited greater acidification during fermentation, particularly at 30°C. The 100% Kernza group showed the steepest decline in pH, supporting the hypothesis that metabolic by-products accumulate more rapidly in high-fiber substrates. However, the relatively stable pH observed in the 75:25 blend at 25°C may contribute to its enhanced fermentation profile. A stable acid-base environment likely supports consistent enzymatic activity and yeast variability over the full fermentation period.

From a process optimization perspective, the results suggest that Kernza is most effective as a co-fermentation substrate rather than a standalone feedstock. While its use alone may require enzymatic pretreatment to improve sugar accessibility, low-to-moderate inclusion in corn-based systems can enhance ethanol yield without compromising fermentation stability. This aligns with broader trends in bioprocessing that emphasize substrate synergy and ecological sustainability.

Importantly, these results also challenge the assumption that higher fermentation temperature may reflect enhanced ethanol production. Instead, temperature effects appear highly strain- and substrate-dependent, requiring tailored protocols that balance enzyme performance with microbial resilience.

TABLE 6 | Quantified carbohydrate total based on literature for corn and Kernza.

Blend	Total carbohydrates (g/200 mL)
100% Corn	1.492
75_25% C_K	1.4635
50_50% C_K	1.435
25_75% C_K	1.4065
100% Kernza	1.378

Acknowledgments

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

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TABLE 7 | Depiction of pH changes across all groups and their statistical significance, (*) being significant.

Temperature	Blend	Initial pH	Final pH	Mean ΔpH	Prob < *t*
30	JW (Control)	7.01	6.37	−0.63	0.0358*
30	100% Corn	5.95	3.84	−2.11	0.0001*
30	75_25% C_K	6.15	3.97	−2.18	0.00007*
30	50_50% C_K	6.05	4.06	−1.99	0.0056*
30	25_75% C_K	5.98	3.90	−2.08	0.0001*
30	100% Kernza	6.22	3.87	−2.34	0.0001*
25	JW (Control)	6.73	6.62	−0.1033	0.1971
25	100% Corn	5.86	3.93	−1.93	0.0014*
25	75_25% C_K	5.84	4.44	−1.40	0.0843
25	50_50% C_K	5.90	4.46	−1.43	0.0674
25	25_75% C_K	6.17	4.34	−1.83	0.0127*
25	100% Kernza	6.23	4.4	−1.83	0.0379*

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