Prisma Sains: Jurnal Pengkajian Ilmu dan Pembelajaran Matematika dan IPA IKIP Mataram
https://e-journal.undikma.ac.id/index.php/prismasains/index
e-mail: prismasains.pkpsm@gmail.com

October 2026. Vol. 14, No. 4
p-ISSN: 2338-4530
e-ISSN: 2540-7899
pp. 2814-2830

Study of Ethanol Level from Liquid Starter Through Spontaneous Fermentation

[1]*Hafiz Iqbal Maulana, [2]Nugraha Edhi Suyatma, [2]Dase Hunaefi, [3]Ajeng Erika Prihastuti Haskito

[1]The Assessment Institute for Foods, Drugs and Cosmetics – The Indonesia Council of Ulama (AIFDC-ICU), Indonesia.
[2]Department of Food Science and Technology, Bogor Agriculture University, Bogor, Indonesia.
[3]Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia.

*Corresponding author e-mail: maulana.hafiz88@gmail.com

Received: September 2026; Revised: October 2026; Published: October 2026

Abstract

Spontaneously fermented liquid starters have attracted interest as potential alternatives to commercial yeast in bread production. Raisins provide fermentable sugars and naturally occurring microorganisms that may support fermentation. This study aimed to evaluate the effects of storage temperature and duration on ethanol accumulation in a spontaneously fermented raisin-based liquid starter. The starter was prepared from raisins, water, and sugar and stored at room temperature (26 °C) or refrigerated temperature (9 °C). Ethanol concentrations were measured at an initial baseline (0 h) and after 24, 48, 72, 120, and 168 h, with three experimental replicates per treatment combination. Ethanol was quantified using gas chromatography–flame ionization detection (GC–FID), with n-propanol as the internal standard. The reported two-way analysis of variance (ANOVA), based on a 2 × 5 factorial arrangement excluding the shared baseline, indicated significant effects of storage temperature, duration, and their interaction on ethanol concentration ($P < 0.001$). At room temperature, ethanol was first detected at 72 h (0.028% w/w), reached its highest measured concentration at 120 h (0.263% w/w), and declined at 168 h (0.057% w/w). These concentrations were assigned to different groups by Duncan's multiple range test ($P < 0.05$). In contrast, ethanol remained undetected throughout refrigerated storage. These findings demonstrate distinct temperature-dependent patterns of ethanol accumulation under the investigated conditions. However, the highest observed ethanol concentration does not establish an optimal fermentation duration, and ethanol measurements alone cannot determine the starter's suitability for bread production because leavening performance was not evaluated.

Keywords: Liquid starter; Raisin; Spontaneous fermentation; Ethanol; Storage temperature.

How to Cite: Maulana, H. I., Suyatma, N. E., Hunaefi, D., & Haskito, A. E. P. (2026). Study of Ethanol Level from Liquid Starter Through Spontaneous Fermentation. *Prisma Sains: Jurnal Pengkajian Ilmu Dan Pembelajaran Matematika Dan IPA IKIP Mataram*, *14*(4), 2814–2830. https://doi.org/10.33394/j-ps.v14i4.22849

https://doi.org/10.33394/j-ps.v14i4.22849

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INTRODUCTION

Consumer expectations, purchasing power, and lifestyles regarding the variety and availability of food options continue to evolve alongside rising per capita income in developing nations (Hui & Evranuz, 2012). These changes have contributed to the growing demand for convenient food products with desirable sensory characteristics and diverse functional attributes. Bread is a highly popular food product due to its convenience and widespread familiarity among the public (Sarofa et al., 2014). In bread production, natural fermentation has attracted considerable interest because it can contribute to the development of distinctive flavor, aroma, and texture characteristics (De Vuyst & Neysens, 2005; Poutanen et al., 2014). Natural fermentation relies on the activity of microorganisms originating from raw materials and the environment; consequently, the characteristics of the final product are influenced by the microbial composition and fermentation conditions (De Vuyst et al., 2014, 2016). Understanding the factors that influence microbial activity and metabolite formation is

therefore important for characterizing naturally fermented starters and their potential applications in bread production.

One alternative for supporting the fermentation process in bread making is the use of a natural liquid starter. Such starters can be prepared through spontaneous fermentation using microorganisms naturally associated with fruits, dried fruits, cereals, or vegetables, without the deliberate addition of commercial yeast. Unlike commercial yeast, which relies on selected cultures, spontaneous fermentation does not always involve a uniform initial composition or quantity of microorganisms. Changes in the microbial community composition during fermentation can be influenced by nutrient availability, environmental conditions, and interactions among microorganisms (Fleet, 2003; Jolly et al., 2014; Mudoor Sooresh et al., 2023). These factors may contribute to variations in microbial growth, substrate utilization, and metabolite accumulation during fermentation. Consequently, characterizing the fermentation process under defined conditions is essential for understanding the biochemical changes occurring in natural liquid starters.

Raisins are a potential ingredient for producing liquid starters because they contain sugars that can serve as fermentation substrates. Derived from dried grapes (*Vitis vinifera* L.), raisins are easily obtained, available in convenient packaging, and relatively affordable (Rietschier et al., 2011). Their sugar content is reported to reach approximately 60%, primarily in the form of glucose and fructose, which provides sweetness and has the potential to support the activity of fermentative microorganisms (Rivero-Cruz et al., 2008). Raisins are also utilized in various food products, including bread, confectionery, snacks, and breakfast cereals (Carughi et al., 2021). In addition to providing fermentable sugars, raisins may introduce naturally occurring microorganisms into the soaking medium, thereby supporting spontaneous fermentation. Hio et al. (2025) demonstrated that raisin-soaked water can undergo spontaneous alcoholic fermentation driven by yeasts originating from the raisins, including both *Saccharomyces* and non-*Saccharomyces* groups. During fermentation, ethanol production is accompanied by a decrease in glucose concentration, indicating the utilization of sugars by the microorganisms. These findings establish the potential of raisin-soak water as a substrate for spontaneous alcoholic fermentation and provide a basis for further investigation of ethanol formation under different fermentation conditions.

Ethanol is a metabolite produced through the fermentation of sugars by yeast. Under fermentative conditions, sugars are metabolized via glycolysis and subsequently converted into ethanol and carbon dioxide (Walker & Stewart, 2016). Ethanol formation during fermentation is influenced by yeast activity, the availability of fermentable substrates, and process conditions, particularly temperature and fermentation duration. Temperature fluctuations can affect yeast metabolic activity and ethanol formation kinetics, resulting in varying ethanol concentrations under different fermentation conditions (Nolasco-Hipolito et al., 2023). Furthermore, interactions between microorganisms, such as competition and metabolic synergy, can influence microbial community composition, substrate utilization, and metabolite formation during fermentation. Consequently, spontaneous fermentation can yield distinct metabolite profiles and concentrations, even when using similar raw materials (Nikoloudaki et al., 2024). Monitoring ethanol concentration can therefore provide information about the temporal pattern of metabolite accumulation in spontaneously fermented liquid starters. Nevertheless, ethanol concentration represents only one aspect of fermentation activity and does not independently establish starter quality or bread-leavening performance.

Temperature is a key factor influencing yeast metabolic activity and growth during fermentation. Its effects on ethanol production depend on the physiological characteristics of the microorganisms and the prevailing fermentation conditions (Panda & Maiti, 2024; Shirvanyan, 2023). Conversely, temperatures exceeding the yeast's tolerance range can induce cellular stress, disrupt protein and cell membrane functions, and reduce fermentation efficiency (Postaru et al., 2023). Storage temperature may also influence the metabolic activity and

composition of microbial communities in natural starters. However, the temperature conducive to active fermentation is not necessarily the same as that suitable for starter storage. Storage at room temperature allows fermentation activity to continue, whereas cold temperatures generally slow microbial activity and help preserve the starter, though the specific response depends on the type of microorganism and storage conditions (Coronas et al., 2025). Comparing room-temperature and refrigerated conditions can therefore help characterize differences in ethanol accumulation during the storage of spontaneously fermented raisin-based liquid starters.

In addition to temperature, storage duration can potentially influence ethanol levels, as fermentation activity may continue as long as the yeast remains active and fermentable substrates are available. During the initial stages of fermentation, sugar utilization is typically accompanied by an increase in ethanol levels; however, this increase does not always proceed linearly. Ethanol accumulation can exert physiological stress on the yeast and disrupt cellular activity, thereby potentially affecting the continued production of ethanol (Sahana et al., 2024). Furthermore, shifts in the microbial community composition during spontaneous fermentation can alter the metabolite profiles produced at different stages of the process (Shibo et al., 2024). Accordingly, ethanol concentration may change throughout the fermentation period rather than increase continuously. Monitoring ethanol at successive time intervals is therefore necessary to describe its accumulation pattern and determine whether the effects of storage temperature differ over time.

Previous studies have established that raisin-soak water can support spontaneous alcoholic fermentation and that environmental conditions influence microbial activity and ethanol formation. In particular, Hio et al. (2025) demonstrated spontaneous ethanol production in raisin water and examined the influence of fermentation conditions. However, a more specific characterization of ethanol concentration across successive storage intervals under room-temperature and refrigerated conditions remains relevant for understanding the behavior of raisin-based liquid starters. This study therefore focuses on the time-dependent ethanol profile of a defined raisin-based liquid starter formulation under these two contrasting storage conditions. Its contribution lies in the comparative characterization of ethanol accumulation over the observation period, rather than in establishing spontaneous fermentation of raisin water as a previously unreported phenomenon.

This study aimed to evaluate the effects of storage temperature and duration on ethanol content in a raisin-based liquid starter produced through spontaneous fermentation. The starter was prepared by mixing raisins, water, and sugar and was maintained at room temperature (25–27 °C) or refrigerated temperature (8–10 °C). Ethanol content was evaluated using an initial measurement (0 h) and subsequent observations at 24, 48, 72, 120, and 168 h. The study examined whether ethanol concentrations differed between the two storage temperatures, changed over time, and exhibited temperature-dependent temporal patterns. By describing these differences, the findings provide empirical information on ethanol accumulation in raisin-based liquid starters under the investigated conditions and a basis for further studies assessing their potential technological applications in bread making.

METHOD

Materials and Experimental Conditions

Raisins and granulated sugar were purchased from a local market in Bogor, Indonesia. Drinking water was used to prepare the raisin-based liquid starter. Ethanol and n-propanol of chromatographic grade were used for gas chromatography analysis. The experiment was conducted using glass jars with a nominal capacity of 330–350 mL. Fermentation was carried out spontaneously without the addition of commercial yeast. This approach is consistent with the spontaneous fermentation of raisin water reported by Hio et al. (2025), who demonstrated that naturally occurring microorganisms associated with raisins can support alcoholic fermentation. Two storage-temperature conditions were investigated: room temperature (25–

27 °C) and refrigerated temperature (8–10 °C). The temperatures were monitored using a thermometer throughout the observation period.

Preparation of the Raisin-Based Liquid Starter

The glass jars were washed with soap and water and subsequently heated in an oven at approximately 100 °C for 5 min. Each jar was filled with 200 mL of drinking water, 10 g of raisins, and 5 g of granulated sugar. The mixture was stirred until the sugar had dissolved and then left uncovered for 1 h before the jar was loosely closed to allow the release of fermentation gases. The prepared mixtures were stored under the designated temperature conditions. The room-temperature samples were kept in a Styrofoam container, whereas the refrigerated samples were stored in a refrigerated cabinet maintained at 8–10 °C. The liquid starter was allowed to undergo spontaneous fermentation, and samples were collected according to the predetermined observation schedule.

Experimental Design and Sampling

The experiment comprised 11 treatment combinations, consisting of a shared initial control (0 h) and a 2 × 5 factorial arrangement of two storage temperatures and five subsequent observation times. The observation period included five sampling times: 24, 48, 72, 120, and 168 h. The two storage temperatures were room temperature (25–27 °C) and refrigerated temperature (8–10 °C). Each treatment combination was represented by three experimental replicates, resulting in 33 experimental units. The initial 0-h control was shared between the two storage-temperature conditions and was therefore counted only once in the total number of experimental units. The experimental design and treatment codes are presented in Table 1.

Table 1. Experimental Design and Total Number of Experimental Units for Raisin Liquid Yeast

No.	Storage Temperature	Fermentation/ Obs. Time (hours)	Treatment Code	Replicates
1	Room temperature (25–27 °C)	0*	R0	3
2	Room temperature (25–27 °C)	24	R24	3
3	Room temperature (25–27 °C)	48	R48	3
4	Room temperature (25–27 °C)	72	R72	3
5	Room temperature (25–27 °C)	120	R120	3
6	Room temperature (25–27 °C)	168	R168	3
7	Cold temperature (8–10 °C)	24	D24	3
8	Cold temperature (8–10 °C)	48	D48	3
9	Cold temperature (8–10 °C)	72	D72	3
10	Cold temperature (8–10 °C)	120	D120	3
11	Cold temperature (8–10 °C)	168	D168	3
Total			11 treatment combinations	33 experimental units

Determination of Ethanol Content by GC–FID

Ethanol content was determined using a Thermo Scientific Trace 1310 gas chromatograph equipped with a flame ionization detector (GC–FID). GC–FID is an established technique for the quantitative determination of ethanol and other volatile alcohols in fermented food matrices (Kim et al., 2022; Mansur et al., 2022). The technique has also been applied to the determination of fermentation products and volatile compounds in other matrices (Charapitsa et al., 2013; Lin et al., 2014). Separation was performed using an Rtx-1301 column with an Integra-Guard, consisting of a stationary phase of 6% cyanopropylphenyl and 94% dimethyl polysiloxane. The column dimensions were 30 m in length, 0.53 mm internal diameter, and 3 µm film thickness.

Ethanol calibration standards were prepared at concentrations of 0, 0.02, 0.04, 0.06, and 0.08% (w/v). n-Propanol at 0.5% (w/v) was used as the internal standard. The standards were diluted to the required volume with distilled water and homogenized before analysis. A volume

of 0.5 µL of each standard was injected into the GC–FID system. The ethanol-to-n-propanol peak-area ratio was used to construct the calibration curve against ethanol concentration. Internal-standard calibration is a commonly used approach in quantitative gas chromatography, while the reliability of chromatographic measurements depends on calibration performance and analytical validation (Thompson et al., 2002; Charapitsa et al., 2019).

For sample preparation, 2 g of liquid starter was weighed into a distillation flask, and n-propanol was added as the internal standard at 0.5% (w/w) of the sample. Distilled water was then added to less than half the flask volume. The mixture was distilled using a heating mantle while cooling water circulated through the condenser. The distillate was collected in a 25-mL volumetric flask. After collection, the distillate was brought to the final volume with distilled water and homogenized.

A 0.5-µL aliquot of the prepared sample was injected into the GC–FID system, with the injector temperature set at 180 °C. Ethanol was identified based on its retention time, reported as approximately 6.100 in the original analytical procedure. Each sample was injected in triplicate. Ethanol concentration in the samples was determined from the calibration relationship between ethanol concentration and the ethanol-to-n-propanol peak-area ratio. The final ethanol concentrations were reported as percentages by weight (% w/w).

The analytical performance of chromatographic methods is commonly assessed using calibration characteristics, accuracy, precision, selectivity, and the applicable concentration range (Horwitz, 1982; Thompson et al., 2002). Detection and quantification limits are particularly important when evaluating low analyte concentrations and interpreting measurements that fall below the analytical detection threshold (Currie, 1999; Shrivastava & Gupta, 2011). These characteristics have also been considered in published GC–FID methods for ethanol determination in fermented foods (Kim et al., 2022; Mansur et al., 2022).

Analytical precision was considered in relation to replicate GC–FID measurements. Horwitz and Albert (2006) described concentration-dependent criteria for evaluating analytical method performance, including the distinction between different precision characteristics. The triplicate injections performed for each sample represented technical analytical measurements and were distinct from the experimental replicates assigned to each treatment combination.

Statistical Analysis

The experimental data were organized according to storage temperature, observation time, and experimental replicate. Descriptive statistics were used to summarize ethanol concentrations for each treatment combination, with results expressed as mean ± standard deviation (SD). The initial measurement (0 h) served as a shared baseline control and was excluded from the factorial analysis of variance. The effects of storage temperature, observation time, and their interaction on ethanol content were evaluated using two-way analysis of variance (ANOVA) based on a 2 × 5 factorial arrangement comprising 30 experimental units. Post hoc comparisons among treatment means were conducted using Duncan's multiple range test (Duncan, 1955), with statistical significance established at $P < 0.05$.

Ethanol concentrations reported as not detected (ND) were interpreted with caution because measurements below the analytical detection limit do not necessarily indicate the complete absence of ethanol. Baccarelli et al. (2005) emphasized the importance of appropriate statistical treatment of non-detectable observations to minimize potential bias in data interpretation. Accordingly, the presence of ND values was considered a limitation when interpreting the reported ANOVA and post hoc comparisons. Pearson correlation analysis was also used to examine associations between ethanol content and supporting fermentation parameters, including pH, yeast count, and °Brix. Correlation coefficients were interpreted as measures of statistical association rather than evidence of causal relationships.

RESULTS AND DISCUSSION

Characteristics of the Raisin-Based Liquid Starter

The raisin-based liquid starter was prepared in glass jars used as fermentation vessels (Figure 1). The starter was produced through spontaneous fermentation without the addition of commercial yeast, allowing naturally occurring microorganisms associated with the raw materials to contribute to fermentation. The total sugar content of the mixture was reported as 4.95%, derived from 5 g of added sugar (2.44%) and 10 g of raisins (2.51%). The raisins contained 51.55 g/100 g total sugar and 14.09 g/100 g moisture. Their total yeast count was below 1.5×10^2 CFU/g, with an estimated count of 2.0×10^1 CFU/g. These initial characteristics indicate that the fermentation substrate contained available sugars and a relatively low initial yeast population, both of which are relevant to subsequent microbial activity and metabolite formation.



Figure 1. Preparation of liquid starter (A) and analysis process of liquid starter as a control (B)

The experiment involved two storage temperatures, room temperature (26 ± 1 °C) and refrigerated temperature (9 ± 1 °C), and five observation times following the initial measurement (24, 48, 72, 120, and 168 h), with three replicates per treatment combination. The initial (0 h) measurement served as a shared control for both temperature conditions, resulting in 10 temperature–time combinations and one control, or 33 experimental units. Because the same initial control was used for both temperature conditions, its measurements were reported only once to avoid duplication of the baseline data.

Table 2. Baseline (control, 0 h) results of the liquid starter, shared by both storage temperatures (n = 3)

Replicate	Ethanol	pH	Yeast count (log CFU/g)	°Brix
1	ND	4.41	0.699	3.39
2	ND	4.46	0.602	3.40
3	ND	4.50	0.477	3.30
Mean ± SD	ND	4.46 ± 0.05	0.59 ± 0.11	3.36 ± 0.06

ND = not detected.

Initial measurements showed no detectable ethanol in any of the three replicates. The mean pH, yeast count, and °Brix values were 4.46 ± 0.05, 0.59 ± 0.11 log CFU/g, and 3.36 ± 0.06, respectively (Table 2). These measurements establish the initial physicochemical and microbiological conditions against which changes during fermentation can be described.

Ethanol was quantified using GC–FID with n-propanol as the internal standard. The calibration curve, prepared using ethanol standards of 0, 0.02, 0.04, 0.06, and 0.08% (w/v), was described by the equation $y = 17.668x - 0.0011$, with a coefficient of determination (R^2) of 0.9992. The internal standard eluted at approximately 10.74 min, with an asymmetry factor of 1.07 and approximately 200,848 theoretical plates. No visible ethanol peak was observed in the chromatogram of the room-temperature sample collected after 24 h.

The relative standard deviations of the replicate injections were 0.29%, 0.69%, and 0.09% for the room-temperature samples collected at 72, 120, and 168 h, respectively. These

values were below two-thirds of the corresponding Horwitz coefficients of variation (4.58%, 3.25%, and 4.11%). These results describe the reported precision of the chromatographic measurements. However, analytical injection precision should be distinguished from variability among independent fermentation replicates. Ethanol concentrations were expressed as percentages by weight (% w/w). Samples designated as ND were those in which ethanol was not detected by the analytical method; this designation does not establish that ethanol was completely absent.

Spontaneous fermentation depends on the metabolic activity of microorganisms naturally associated with the substrate and the surrounding environment. In raisin-based liquid starters, dissolved sugars can serve as substrates for yeast metabolism, resulting in the formation of ethanol and carbon dioxide. Hio et al. (2025) demonstrated that raisin-soak water supports spontaneous alcoholic fermentation involving *Saccharomyces* and non-*Saccharomyces* yeasts. Similarly, Tesfaw and Assefa (2014) reported that yeast growth and ethanol production are influenced by several environmental and nutritional factors, including temperature, pH, initial sugar concentration, oxygen availability, and inhibitory compounds. These findings provide a biological basis for examining ethanol accumulation under different storage conditions. Nevertheless, the specific microorganisms responsible for ethanol production in the present starter were not identified.

Effects of Storage Temperature and Duration on Ethanol Content

The effects of storage temperature and observation time on ethanol concentration were evaluated using a 2 × 5 factorial analysis involving 30 experimental units, with the shared initial control excluded from the factorial model. The reported ANOVA indicated significant effects of storage temperature, observation time, and their interaction on ethanol content ($P < 0.001$; Table 3).

Table 3. Analysis of variance for ethanol content (2 × 5 factorial, n = 30)

Source	DF	Sum of squares	Mean square	F	Pr > F
Temperature	1	0.0365	0.0365	2,327.41	< 0.001
Time	4	0.0736	0.0184	1,172.15	< 0.001
Temperature × Time	4	0.0736	0.0184	1,172.15	< 0.001
Error	20	0.0003	0.000016	–	–
Total	29	0.1841	–	–	–

The significant interaction indicates that the observed effect of storage temperature on ethanol concentration depended on the observation time. Consequently, the temperature effect should be interpreted in relation to the temporal changes rather than exclusively through the main effects. The sums of squares and F values for observation time and the temperature–time interaction were identical because ethanol was not detected in any refrigerated treatment. Accordingly, the observed time-dependent variation in ethanol concentration was restricted to the room-temperature treatments.

Interpretation of these ANOVA results requires caution because seven of the ten factorial treatment combinations contained only ND observations, while the detectable concentrations showed relatively small within-group variation. This pattern raises concerns about variance homogeneity and the treatment of non-detectable measurements in the statistical model. Therefore, the reported significance levels should be interpreted alongside the observed concentration patterns and the limitations of the analytical data.

Temperature influences microbial growth, enzymatic activity, and the metabolic conversion of fermentable sugars into ethanol. Tesfaw and Assefa (2014) reported that fermentation efficiency depends on environmental conditions, particularly temperature and substrate availability. Hio et al. (2025) also observed greater ethanol production during spontaneous fermentation of raisin water at 25–30 °C than at 15–20 °C under their experimental conditions. These findings are consistent with the greater ethanol accumulation observed at

room temperature in the present study. However, because microbial species and their metabolic activities were not characterized, the physiological mechanisms underlying the temperature-dependent differences cannot be established directly.

Table 4. Ethanol content (% w/w, mean ± SD, n = 3) of the liquid starter and Duncan grouping ($P < 0.05$)

Temperature	0 h	24 h	48 h	72 h	120 h	168 h
Room (26 °C)	ND (D)	ND (D)	ND (D)	0.028 ± 0.001 (C)	0.263 ± 0.013 (A)	0.057 ± 0.001 (B)
Cold (9 °C)	ND (d)	ND (d)	ND (d)	ND (d)	ND (d)	ND (d)

Different letters indicate significant differences. ND = not detected.

At room temperature, ethanol was first detected at 72 h (0.028%), increased to 0.263% at 120 h (95% CI: 0.232–0.295%), and declined to 0.057% at 168 h (95% CI: 0.056–0.059%) (Table 4; Figure 2). This non-linear pattern indicates that ethanol accumulation varied throughout fermentation. Duncan's multiple range test assigned these concentrations to significantly different groups (C, A, and B), confirming differences among observation times. Although the highest ethanol concentration occurred at 120 h, this finding does not establish an optimal fermentation duration.

In contrast, ethanol remained undetected throughout refrigerated storage (9 °C), including at 168 h, indicating concentrations below the method's detection capability rather than complete inhibition of fermentation. The relatively low maximum ethanol concentration (0.263%) may reflect the limited initial yeast population, substrate characteristics, and fermentation conditions. Fernandes et al. (2022) emphasized that ethanol production by *Saccharomyces cerevisiae* depends on environmental conditions and microbial metabolic characteristics. However, changes in fermentable sugars and microbial composition were not directly investigated. Therefore, the observed ethanol dynamics should be interpreted within the specific starter formulation, storage temperatures, and observation period examined, without generalizing these findings to other fermentation systems.

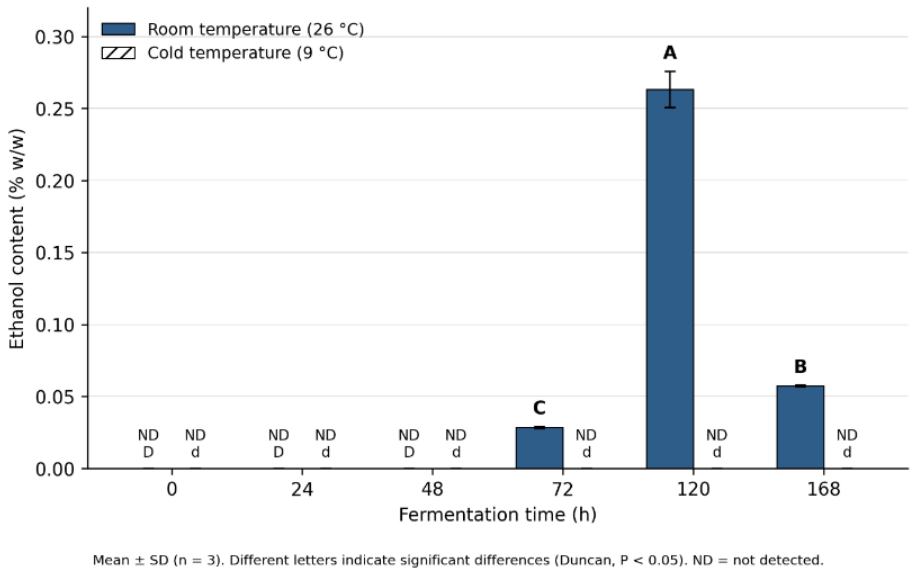

Figure 2. Ethanol content (% w/w, mean ± SD) at room and cold temperature over 168 h, with Duncan letters.

Changes in pH, Yeast Count, and °Brix During Fermentation

Changes in pH, yeast count, and °Brix were evaluated as supporting indicators of fermentation dynamics (Table 5). These parameters provide additional information on the physicochemical and microbiological changes occurring during storage, although they do not independently establish the mechanisms responsible for ethanol formation.

Table 5. pH, yeast count (log CFU/g), and °Brix of the liquid starter (mean ± SD, n = 3)

Temperature	Hour	pH	Yeast count (log CFU/g)	°Brix
Control	0	4.46 ± 0.05	0.59 ± 0.11	3.36 ± 0.06
Room	24	4.36 ± 0.01	2.07 ± 1.04	4.98 ± 0.13
Room	48	4.14 ± 0.03	3.61 ± 0.74	5.25 ± 0.02
Room	72	3.97 ± 0.06	5.91 ± 1.97	5.57 ± 0.06
Room	120	3.90 ± 0.10	4.33 ± 1.22	5.78 ± 0.06
Room	168	3.83 ± 0.06	3.66 ± 1.02	6.19 ± 0.02
Cold	24	4.41 ± 0.09	1.13 ± 0.41	4.96 ± 0.13
Cold	48	4.30 ± 0.10	1.19 ± 0.36	5.22 ± 0.07
Cold	72	4.24 ± 0.02	1.78 ± 0.72	5.41 ± 0.10
Cold	120	4.20 ± 0.03	3.19 ± 0.69	5.69 ± 0.04
Cold	168	4.14 ± 0.04	3.95 ± 1.02	6.04 ± 0.02

The yeast population increased during the early stages of room-temperature storage, reaching its highest recorded value of 5.91 log CFU/g at 72 h, before decreasing to 4.33 and 3.66 log CFU/g at 120 and 168 h, respectively. Under refrigerated conditions, the yeast count increased more gradually, reaching 1.78 log CFU/g at 72 h and 3.95 log CFU/g at 168 h.

The greater yeast population observed at room temperature during the early fermentation period is consistent with the earlier detection and subsequent accumulation of ethanol under this condition. Nevertheless, the maximum yeast count occurred at 72 h, whereas the highest ethanol concentration was observed at 120 h. This temporal difference suggests that ethanol accumulation did not directly coincide with the maximum measured yeast population.

The pH decreased progressively under both temperature conditions. At room temperature, pH declined from the initial value of 4.46 to 3.83 after 168 h, whereas refrigerated storage resulted in a pH of 4.14 at the same observation time. The greater reduction in pH at room temperature indicates more pronounced acidification under this condition. However, the specific organic acids responsible for the pH changes were not quantified, preventing attribution of the observed acidification to particular microbial metabolic pathways.

The reported ANOVA showed significant effects of storage temperature, observation time, and their interaction on pH ($F = 98.73$, 41.76, and 4.92; $P < 0.001$, < 0.001, and 0.006, respectively). Significant effects were also reported for yeast count ($F = 20.14$, 6.10, and 4.07; $P < 0.001$, 0.002, and 0.014, respectively). For °Brix, storage temperature ($F = 9.95$; $P = 0.005$) and observation time ($F = 199.08$; $P < 0.001$) were significant, whereas their interaction was not significant ($F = 1.13$; $P = 0.373$).

In contrast to the decline typically expected when soluble sugars are consumed during fermentation, °Brix increased progressively under both storage conditions. At room temperature, °Brix increased from 3.36 at 0 h to 6.19 at 168 h, while refrigerated samples reached 6.04 at 168 h. This pattern may reflect the continuing dissolution of soluble compounds from the raisins into the surrounding water. Because °Brix represents the refractometric response of dissolved substances rather than a direct measurement of individual fermentable sugars, the observed increase cannot be interpreted as evidence of increasing glucose or fructose concentrations.

Pearson correlation analysis showed a moderate negative association between ethanol concentration and pH ($r = -0.59$, $P < 0.001$) and a weak positive association between ethanol concentration and yeast count ($r = 0.36$, $P = 0.038$). The association between ethanol concentration and °Brix was not statistically significant ($r = 0.30$, $P = 0.092$; $n = 33$).

These correlations indicate that greater ethanol concentrations tended to occur alongside lower pH values and higher yeast counts within the pooled dataset. However, because the analysis combined observations from different temperatures and storage times, the associations may partly reflect common responses to the experimental conditions. The correlations therefore should not be interpreted as evidence that changes in pH or yeast population directly caused changes in ethanol concentration.

The increase in yeast counts and the decrease in pH during refrigerated storage further indicate that measurable biological and physicochemical changes continued under cold conditions despite the absence of detectable ethanol. Consequently, refrigerated storage should be interpreted as a condition associated with reduced detectable ethanol accumulation rather than complete cessation of fermentation activity.

Interpretation of the Decline in Ethanol Concentration During Extended Storage

One of the notable findings of this study was the substantial decline in ethanol concentration from 0.263% at 120 h to 0.057% at 168 h under room-temperature storage. This pattern indicates that ethanol accumulation was not continuous throughout the observation period, suggesting changes in the balance between ethanol production and processes that may reduce its concentration during extended fermentation. Ethanol dynamics in spontaneously fermented systems can be influenced by substrate availability, yeast metabolic activity, microbial interactions, and environmental conditions (Stanley et al., 2010a; Huang et al., 2023). However, the present study measured ethanol concentration at discrete observation times and did not directly determine the rates of ethanol production or removal. Therefore, the observed decline should be interpreted as a change in ethanol concentration rather than definitive evidence of a particular metabolic mechanism.

Changes in yeast physiology and substrate utilization may contribute to variations in ethanol accumulation during prolonged fermentation. Yeast metabolic activity depends on the availability of fermentable sugars and the ability of cells to maintain physiological functions under changing environmental conditions. Stanley et al. (2010a) described how ethanol exposure can influence yeast stress responses and cellular performance, while Stanley et al. (2010b) demonstrated that ethanol tolerance involves changes in gene expression and cellular adaptation. Nevertheless, ethanol-induced physiological stress cannot be established as the cause of the decline observed in the present study, particularly because the measured ethanol concentrations were relatively low and the physiological responses of the indigenous yeasts were not investigated. Consequently, changes in microbial activity and substrate availability remain possible, but unconfirmed, explanations.

Another potential mechanism is the microbial oxidation of ethanol into acetic acid. Acetic acid bacteria can oxidize ethanol through aerobic metabolic pathways, thereby contributing to changes in ethanol and organic acid concentrations during fermentation. This process has been widely described in studies of acetic acid bacteria and vinegar production (Gullo & Giudici, 2008; Gullo et al., 2014; Gomes et al., 2018). Lynch et al. (2019) further emphasized the role of oxidative metabolism in the transformation of ethanol into acetic acid, while De Roos and De Vuyst (2018) highlighted the occurrence and functional diversity of acetic acid bacteria in naturally fermented foods and beverages. These findings support the biological plausibility of ethanol oxidation in mixed microbial fermentation systems. However, because oxygen availability, acetic acid concentration, and the presence of acetic acid bacteria were not determined in the present study, ethanol oxidation cannot be confirmed as the mechanism responsible for the observed decline.

Changes in microbial community composition may also influence ethanol accumulation during spontaneous fermentation. Unlike fermentation processes initiated with selected microbial cultures, spontaneous fermentation involves naturally occurring microbial communities whose composition and metabolic activity can change throughout the process. Huang et al. (2023) demonstrated that microbial succession during spontaneous grape fermentation was associated with changes in volatile compound profiles. Similarly, De Vuyst et al. (2023) emphasized that interactions among yeasts and bacteria, together with substrate characteristics and fermentation conditions, influence the metabolic functionality of naturally fermented starters. These findings suggest that microbial succession may contribute to variations in fermentation metabolites. Nevertheless, the present study did not identify the yeast or bacterial species present during storage, preventing a direct association between microbial

community changes and declining ethanol concentrations.

In addition to biological mechanisms, physical losses may influence the measured ethanol concentration. Williams (1983) demonstrated that ethanol can be lost through evaporation during alcoholic fermentation and that evaporative losses depend on fermentation conditions. Therefore, ethanol volatilization represents another possible explanation for the decline observed during extended storage, particularly if gas exchange occurred through the fermentation container. However, the present study did not quantify evaporative losses or directly monitor gas exchange. Consequently, the contribution of ethanol volatilization remains uncertain.

Taken together, the available literature indicates that ethanol concentration during spontaneous fermentation may reflect the combined influence of microbial metabolism, substrate availability, oxidation, and physical losses. Nevertheless, these mechanisms cannot be distinguished using the measurements obtained in the present study. The absence of residual sugar measurements, organic acid profiles, microbial identification, and oxygen monitoring limits the identification of the processes responsible for the decrease in ethanol concentration between 120 and 168 h. Future investigations incorporating these measurements would provide a more comprehensive understanding of ethanol accumulation and decline in raisin-based liquid starters.

Implications and Limitations of the Study

The results demonstrate that storage temperature and duration were associated with distinct patterns of ethanol accumulation in the spontaneously fermented raisin-based liquid starter. Under room-temperature storage, ethanol was first detected at 72 h, reached its highest measured concentration of 0.263% at 120 h, and declined to 0.057% at 168 h. In contrast, ethanol remained undetected throughout the refrigerated storage period. These findings are consistent with the observation of Hio et al. (2025), who demonstrated that raisin-soak water can support spontaneous alcoholic fermentation involving naturally occurring yeasts. The present study extends the empirical characterization of raisin-based liquid starters by describing ethanol concentrations at successive observation times under two contrasting storage-temperature conditions using a defined formulation.

From a technological perspective, the observed differences suggest that storage conditions should be considered when characterizing ethanol accumulation in spontaneously fermented liquid starters. Nevertheless, the highest concentration measured at 120 h should not be interpreted as evidence of an optimal fermentation duration. The characteristics and functionality of natural starters depend on microbial ecology, substrate composition, and fermentation management. Arora et al. (2021) emphasized that sourdough fermentation performance is influenced by interactions among microorganisms and process conditions, while De Vuyst et al. (2023) highlighted the importance of microbial ecology and fermentation strategies in determining starter functionality. Accordingly, the ethanol profile observed in the present study should be considered specific to the investigated formulation and storage conditions.

Several methodological limitations should be acknowledged. First, the study involved only three experimental replicates per treatment combination and a single starter formulation, limiting the generalizability of the findings. Second, the analytical limits of detection and quantification were not reported. Consequently, measurements classified as not detected cannot be interpreted as confirmed zero ethanol concentrations. Appropriate treatment of observations below analytical detection limits is essential for reliable statistical interpretation, as demonstrated by Baccarelli et al. (2005) and Helsel (2006). Third, the ethanol data exhibited heterogeneous within-group variability, and the statistical treatment of non-detectable observations was not fully documented. These limitations should be considered when interpreting the reported ANOVA and Duncan multiple range comparisons.

Another limitation concerns the incomplete characterization of the microbial and

biochemical changes occurring during fermentation. Although yeast counts, pH, and °Brix were measured, these parameters do not identify the microorganisms responsible for fermentation or directly quantify the utilization of specific fermentable sugars. Moreover, the concentrations of residual glucose, fructose, lactic acid, acetic acid, and other fermentation metabolites were not determined. Studies of spontaneous fermentation have demonstrated that microbial succession and metabolic changes can influence the chemical characteristics of fermented substrates (Huang et al., 2023; De Vuyst et al., 2023). Therefore, the absence of detailed microbial and metabolite analyses restricts mechanistic interpretation of the observed ethanol patterns.

Furthermore, ethanol concentration alone is insufficient to establish the technological suitability of a liquid starter for bread production. Bread-leavening performance depends not only on microbial fermentation but also on carbon dioxide production, dough properties, microbial composition, and processing conditions. Clark et al. (2024) demonstrated that sourdough starter microbiomes can influence the physical and chemical characteristics of wheat bread, including loaf volume and crumb properties. Similarly, Verdonck et al. (2023) reported that bread volume depends on interactions between leavening strategies and processing parameters. De Bondt et al. (2024) further highlighted the importance of considering fermentation conditions, leavening agents, and bread-quality characteristics when evaluating sourdough-based breadmaking. Because the present study did not assess carbon dioxide production, dough expansion, or bread quality, its findings cannot establish the effectiveness of the raisin-based liquid starter as a bread-leavening agent.

Future investigations should therefore integrate microbial identification, residual sugar analysis, organic acid profiling, and measurements of oxygen availability to clarify the mechanisms underlying ethanol accumulation and decline. Analytical procedures should also include validated detection and quantification limits, while statistical approaches should adequately address non-detectable observations. In addition, direct evaluations of carbon dioxide production, dough fermentation, and bread-quality characteristics are recommended to establish the potential application of raisin-based liquid starters in bread production. Such studies would provide a stronger scientific basis for understanding the fermentation characteristics and technological functionality of spontaneously fermented raisin-based starters.

CONCLUSION

Storage temperature and duration influenced ethanol accumulation in the spontaneously fermented raisin-based liquid starter, with the reported statistical analysis indicating significant effects of both factors and their interaction. Under room-temperature storage (26 °C), ethanol was first detected after 72 h (0.028%), reached its highest measured concentration at 120 h (0.263%), and subsequently declined at 168 h (0.057%). The reported Duncan's multiple range test indicated significant differences among these three concentrations ($P < 0.05$). In contrast, ethanol remained undetected throughout refrigerated storage (9 °C) for up to 168 h. These findings demonstrate that ethanol accumulation followed a temperature-dependent temporal pattern under the investigated fermentation conditions.

The study contributes empirical information on ethanol dynamics in a spontaneously fermented raisin-based liquid starter prepared using a defined formulation. However, the highest ethanol concentration observed at 120 h does not necessarily represent the optimal fermentation duration, and the absence of detectable ethanol under refrigerated conditions does not indicate the complete cessation of microbial activity. Furthermore, ethanol concentration alone cannot establish the starter's suitability for bread production because its leavening performance was not evaluated. The findings therefore provide a basis for further investigation of the biochemical, microbiological, and technological characteristics of raisin-based liquid starters.

RECOMMENDATION

Further research should investigate the mechanisms underlying ethanol accumulation and its subsequent decline during extended fermentation of raisin-based liquid starters. Particular attention should be given to changes in residual fermentable sugars, including glucose and fructose, organic acid concentrations, especially acetic and lactic acids, and the composition and dynamics of yeast and bacterial communities. Monitoring oxygen availability and potential ethanol volatilization would also help distinguish between biological and physical processes contributing to changes in ethanol concentration.

Future studies should employ validated analytical procedures with clearly established limits of detection and quantification, appropriate treatment of non-detectable measurements, and experimental designs that support reliable statistical inference. Investigations involving additional starter formulations, storage temperatures, fermentation durations, and independent experimental replicates are recommended to evaluate the reproducibility and generalizability of the observed ethanol patterns.

The technological potential of raisin-based liquid starters should also be assessed through direct bread-making trials. These evaluations should include carbon dioxide production, dough-leavening capacity, fermentation performance, bread volume, crumb structure, and sensory characteristics. Integrating these measurements with microbiological and biochemical analyses would provide a more comprehensive basis for determining suitable fermentation conditions and evaluating the practical application of spontaneously fermented raisin-based liquid starters in bread production.

ACKNOWLEDGMENTS

We would like to express our gratitude to LPPOM MUI and IPB University for facilitating the use of research laboratories during the conduct of this research.

FUNDING INFORMATION

This research received no external funding.

AUTHOR CONTRIBUTIONS STATEMENT

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Hafiz Iqbal Maulana	✓	✓			✓	✓		✓	✓	✓	✓			✓
Nugraha Edhi Suyatma	✓	✓			✓		✓			✓			✓	
Dase Hunaefi	✓	✓		✓	✓	✓	✓			✓				
Ajeng E.P. Haskito			✓			✓		✓		✓	✓			✓

CONFLICT OF INTEREST STATEMENT

Authors state no conflict of interest.

ETHICAL APPROVAL

This study did not involve human participants or experimental animals. Therefore, ethical approval from a human research ethics committee or an animal ethics committee was not required. The study involved the spontaneous fermentation of a raisin-based liquid starter and the subsequent analysis of ethanol concentration.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The data include the experimental results on ethanol concentrations in raisin-based liquid starters under different storage temperatures and fermentation durations, as well as the relevant analytical results obtained using gas chromatography–flame ionization detection (GC–FID).

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