

The Effect of α -Amylase and Glucoamylase Enzyme Concentrations and Fermentation Time on the Characteristics of Bioethanol from Cempedak Seed Flour

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Article Information	ABSTRACT
Article History Received : August 07, 2026 Revised : September 28, 2026 Published : October 02, 2026	Indonesia's reliance on fossil fuels highlights the need for renewable energy sources, including bioethanol derived from non-food biomass. Cempedak seeds (<i>Artocarpus champeden</i> sp.) represent an underutilized tropical agro-industrial waste; however, their high starch content ($\pm 71.8\%$) makes them a promising substrate for bioethanol production. This study aimed to examine the effects of alpha-amylase and glucoamylase enzyme concentration ratios (0.5%:1.5%, 1%:1%, and 1.5%:0.5%) and fermentation durations (2, 3, 4, and 5 days) on the characteristics of cempedak seed flour bioethanol, including total sugar content, alcohol content, moisture content, pH, and density. The study utilized a two-factor Randomized Complete Block Design (RCBD) with two replications. It was analyzed using Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test at a 5% significance level. The concentration ratio of α -amylase and glucoamylase enzymes had a significant effect on total sugar content, alcohol content, moisture content, pH value, and density. Fermentation duration had a significant effect on alcohol content, moisture content, pH, and density, but no significant effect on total sugar content. The treatment with an α -amylase-to-glucoamylase ratio of 0.5%:1.5% and a fermentation duration of 2 days produced the highest alcohol content (46%).
Keywords: Bioethanol; α -Amylase; Glucoamylase; Cempedak; Fermentation time.	

INTRODUCTION

The global energy crisis and environmental issues stemming from reliance on fossil fuels have prompted many countries to develop renewable, environmentally friendly alternative energy sources. Indonesia, as a country with continuously increasing energy consumption, is making serious efforts to meet its national energy demands. According to data from the Ministry of Energy and Mineral Resources, Indonesia's crude oil reserves continue to decline, while fuel demand continues to rise due to economic and population growth. This situation necessitates energy source diversification through the use of renewable resources, including bioethanol (Lestariningsih & Fatimah, 2024)

Bioethanol is a plant-based fuel produced through the fermentation of simple sugars using microorganisms, primarily the yeast *Saccharomyces cerevisiae*. As an alternative fuel, bioethanol offers several advantages over fossil fuels, such as lower greenhouse gas emissions, renewability, and the ability to be produced from various types of biomass. Bioethanol with a

minimum purity of 99.5% (fuel grade) can be used as a fuel blend or as a standalone fuel for motor vehicles (Mgeni et al., 2025).

Bioethanol can be produced from three main types of feedstocks: starch-containing crops, materials with high simple sugar content, and cellulosic materials. High-yielding, abundant staple food crops can compete for food supplies, potentially affecting prices. Simple sugars can be fermented directly without hydrolysis, making the process faster and more energy-efficient, but they are limited by harvest seasons and have a shorter shelf life. Cellulosic materials do not compete with food supplies and are abundant, but they require expensive, complex pretreatment processes (Lestariningsih & Fatimah, 2024). However, the use of primary food crops as bioethanol feedstocks has sparked controversy because it creates competition with food needs. Therefore, exploring alternative non-food feedstocks or agricultural waste has become a highly relevant research focus. One potential feedstock that remains under-explored is cempedak seeds (*Artocarpus champeden sp.*).

The bioethanol production process from starch-containing materials, such as cempedak seed flour, involves two interconnected main stages: enzymatic hydrolysis and fermentation (Arifiyanti et al., 2020). In the enzymatic hydrolysis stage, starch, which is a complex polysaccharide, must first be broken down into simple sugars that can be fermented by yeast. This hydrolysis process requires the synergistic action of two types of enzymes: α -amylase and glucoamylase.

Cempedak seeds (*Artocarpus champeden sp.*) can be used as a raw material for bioethanol production due to their high starch content of approximately 71.8%. Furthermore, cempedak seeds are not a staple food, so their utilization does not disrupt public food supplies. Additionally, cempedak seeds remain underutilized and are generally considered waste. Their high extract content makes cempedak seeds a potential source of bioethanol (Pui et al., 2018).

α -Amylase is an endo-amylase enzyme that acts by randomly cleaving α -1,4 glycosidic bonds within the internal starch chains. The activity of this enzyme yields oligosaccharides of varying sizes, predominantly dextrin, maltotriose, and maltose. Glucoamylase, also known as amyloglucosidase, is an exo-amylase enzyme that functions by sequentially cleaving α -1,4 glycosidic bonds from the non-reducing ends of starch or dextrin chains, thereby releasing free glucose molecules. Additionally, glucoamylase can hydrolyze α -1,6 glycosidic bonds at the branch points of amylopectin, albeit at a slower rate (Cornejo-Ramírez et al., 2018). Enzyme concentration is a critical factor influencing the efficiency of converting starch into simple sugars. If the enzyme concentration is too low, the hydrolysis process will be slow and incomplete, thereby limiting the availability of sugar for fermentation. On the other hand, an excessively high enzyme concentration does not yield a significant increase in output and needlessly raises production costs.

Although several studies have identified the high starch potential of *Artocarpus* seed wastes for bioenergy production, a significant research gap remains regarding the dynamic optimization of dual-enzyme kinetics during the separate hydrolysis and fermentation (SHF) of *Artocarpus champeden* (cempedak) seed flour (Arifiyanti et al., 2020). Existing literature heavily focuses on alternative tropical fruit seeds like jackfruit (*Artocarpus heterophyllus*) and durian (*Durio zibethinus*), evaluating basic acid hydrolysis or single-factor yeast optimization (Ginting et al., 2020). However, cempedak seed flour contains unique structural properties, including a higher proportion of resistant starch (up to 19% depending on thermal pre-treatments), which inherently restricts enzyme accessibility compared to standard starch matrices (Khayrah et al., 2022a). While a multi-enzyme system combining endo-amylase (α -amylase) and exo-amylase (glucoamylase) can induce synergistic effects to accelerate saccharification (Guo et al., 2021). Current research lacks systemic data on how these specific ratios interact within the complex cempedak matrix. Determining this optimal synergistic ratio is critical to minimizing production costs while avoiding enzyme inhibition during hydrolysis. Furthermore, a definitive evaluation of the kinetic boundaries governing glucose release from starch under continuous dual-enzymatic

saccharification remains unaddressed (Yu et al., 2018). This knowledge gap hinders the scalable, cost-effective transformation of this specific agricultural waste into high-yield bioethanol.

Fermentation time is a crucial factor determining how much sugar can be converted into ethanol. At the beginning of fermentation, yeast undergoes an adaptation phase to its new environment. Subsequently, the yeast enters the exponential growth phase, during which its metabolic activity and ethanol production reach their peak levels. Over time, the accumulation of ethanol and the depletion of substrates cause the yeast to enter a stationary phase and ultimately a death phase (Yuliani et al., 2024).

Based on the background discussed above, this study is essential for exploring the potential of cempedak seeds as a bioethanol feedstock and determining the optimal process conditions by varying the concentrations of alpha-amylase and glucoamylase enzymes, as well as the fermentation time. The objective of this research was to determine the effect of the α -amylase to glucoamylase concentration ratio and fermentation time on the physical and chemical characteristics of bioethanol produced from cempedak seed flour. This research is expected to contribute to the scientific development of bioethanol from Indonesian local resources and support national efforts to diversify sustainable energy sources.

RESEARCH METHODS

Material

The materials used in this study were cempedak seed flour, alpha-amylase and glucoamylase enzymes, baker's yeast as the fermentation inoculum, urea and NPK as nutrient sources, acetic acid, 0.1 N NaOH, and distilled water.

Experimental Design and Data Analysis

This study used a two-factor Randomized Complete Block Design (RCBD). RCBD was employed to account for temporal variability across experimental runs conducted on different days (e.g., daily fluctuations in ambient laboratory conditions or fresh enzyme preparation). The first factor was the ratio of alpha-amylase and glucoamylase enzyme concentrations, while the second factor was the fermentation duration. Factor 1 (the alpha-amylase to glucoamylase concentration ratio) consisted of 3 factor levels (0.5%:1.5%, 1%:1%, and 1.5%:0.5%), and Factor 2 (fermentation duration) consisted of 4 factor levels (2 days, 3 days, 4 days, and 5 days). The experiment was arranged into 2 blocks (replicates). Furthermore, each chemical analysis was performed in duplicate. The obtained data were subjected to Analysis of Variance (ANOVA), and significant differences were further evaluated using Duncan's Multiple Range Test at a 5% significance level.

Preparation of Cempedak Seed Flour

The processing of cempedak seed flour refers to Indriyanti Putri & F. Rosida, (2025) with modification. Cempedak seeds separated from the fruit pulp were thoroughly washed until the rinsing water ran clear. The seeds were then steamed for 5–10 minutes at 80–90°C to inactivate enzymes and reduce anti-nutritional compounds. Following steaming, the seeds were briefly cooled and sun-dried naturally at approximately 35–45°C, which typically took 12–24 hours depending on climatic conditions and humidity. Regular stirring was performed every 2–3 hours to ensure uniform drying and prevent spoilage. Once the moisture content dropped to 8–10%, the dried seeds were ground using a feed mill or a high-capacity blender to form a fine flour, which was subsequently sieved to remove coarse particles. Finally, the flour was stored in airtight containers in a cool, dry place.

Hydrolysis

Prepare 100 g of cempedak seed flour. Add water at a ratio of 1:6 (w/v) (100 g flour + 600 mL water), then heat at 90 °C for 30 minutes in a beaker while stirring continuously. Add alpha-amylase enzyme in accordance with the treatment (0.5, 1.0 and 1.5% w/w, based on total

flour weight) to the starch solution. This liquefaction process is conducted for 1 hour at 80 °C. Upon completion of liquefaction, reduce the solution temperature to 60 °C, followed by saccharification with the addition of glucoamylase enzyme in accordance with the treatment (1.5, 1.0 and 0.5% w/w, based on total flour weight). This process is maintained for 1 hour at 60 °C. Incubate the resulting solution until two distinct layers are formed, consisting of a liquid phase and a slurry precipitate (solid phase). Cool the mixture to 30–37 °C, and subsequently analyze the total sugar content.

Fermentation and Distillation.

A total of 500 mL of the hydrolysate was prepared, followed by the addition of 5% (w/w, based on total flour weight) urea (5 g) and 1% (w/w, based on total flour weight) NPK fertilizer (1 g), calculated based on the initial cempedak seed flour weight. The urea and NPK were finely ground, thoroughly mixed into the solution, and transferred into a 1-L simple reactor. Prior to fermentation, the filtrate was sterilized at 121 °C for 15 minutes to eliminate or inactivate pathogenic and competing microorganisms present in the raw material. Baker's yeast (5% w/w based on flour weight, i.e., 5 g) pre-dissolved in a small volume of warm water was added. The pH was adjusted to 4.5–5.0 using acetic acid. Fermentation was subsequently carried out for various incubation periods (2, 3, 4, and 5 days). Following fermentation, the broth was filtered to remove residual yeast. The fermented solution was then distilled using a rotary vacuum evaporator (Scilogex RE 100-s) at 60 °C and 100 rpm. The resulting alcoholic liquid was collected and subjected to a second distillation step at 80 °C for 50 minutes. The purified distillate was subsequently analyzed for total sugar content using a refractometer, alcohol concentration using an alcoholmeter, density using a pycnometer, and total acidity (expressed as acetic acid).

RESULTS AND DISCUSSION

Cempedak is a tropical plant widely grown in tropical regions. Cempedak fruit is an agricultural commodity with promising future prospects, as it serves not only as a food source but can also be projected for industrial applications. The utilization of cempedak seeds as a raw material for bioethanol production offers several advantages, including abundant availability as agricultural waste, non-competition with primary food supplies, high carbohydrate content, and increased economic value post-processing (Khayrah et al., 2022b). Starch in cempedak seeds predominantly consists of two major components, namely amylose and amylopectin, which can be hydrolyzed into glucose for subsequent fermentation. Amylose is a linear polysaccharide composed of glucose units linked by α -(1,4)-glycosidic bonds. In contrast to branched amylopectin, amylose tends to form straight chains that can adopt a helical structure. Amylopectin, on the other hand, is water-insoluble with a highly branched-chain structure. In amylopectin, glucose molecules are linked by α -(1,6)-glycosidic bonds, which are notoriously resistant to cleavage (Putri & Rosida, 2025).

Total sugar content of the hydrolysate (%Brix)

The total sugar content of the hydrolysate is shown in Table 1.

Table 1. The total sugar content of the hydrolysate (%Brix)

Fermentation time (days)	α -Amilase : Glukoamilase			Average
	(0.5 : 1.5)%	(1 : 1)%	(1.5 : 0.5)%	
2	14.00±1.41 ^{cd}	14.00±0.00 ^{cd}	12.00±0.00 ^{abc}	13.33±1.21 ^q
3	14.00±1.41 ^{cd}	13.50±0.70 ^{bcd}	12.00±0.00 ^{abc}	13.17±1.16 ^q
4	13.00±1.41 ^{bcd}	12.00±1.41 ^{abc}	10.00±0.00 ^a	11.67±1.63 ^p
5	11.50±0.70 ^{ab}	15.00±0.00 ^d	13.00±0.00 ^{bcd}	
Average	13.13±1.45 ^y	13.63±1.30 ^y	11.75±1.16 ^x	

Note: Means within columns or rows followed by different letters indicate significant differences based on Duncan's Multiple Range Test at the 5% significance level.

Based on Table 1, the mean total sugar content fluctuated across fermentation periods. The average total sugar content decreased from 13.33 °Brix at 2 days of fermentation to 11.67

°Brix at 4 days. The observed increase in °Brix at day 5 (B4) in treatments A2 (15.00 °Brix) and A3 (13.00 °Brix) can be attributed to residual enzymatic activity. As *Saccharomyces cerevisiae* enters the death phase—driven by the accumulation of toxic ethanol or the depletion of essential micronutrients—the glucose consumption rate by yeast cells virtually ceases.

This pattern aligns with the principles reported by Visca et al., (2020). In their study on glucoamylase optimization in rice washing water, glucose concentration was found to increase proportionally with the dosage of added glucoamylase. This enzyme plays a pivotal role in yielding glucose monomers, which are quantified as total dissolved solids by a refractometer. A higher proportion of glucoamylase facilitates the further breakdown of dextrin molecules—initially generated by α -amylase—into glucose. Because glucose is a readily soluble simple sugar, its accumulation directly elevates the Brix value. Conversely, at lower glucoamylase concentrations, a fraction of dextrin remains unconverted, resulting in a lower total sugar yield (Safriana Safitrie et al., 2015).

The two-way analysis of variance (ANOVA) revealed a significant interaction effect between the enzyme concentration ratio (α -amylase : glucoamylase) and fermentation duration on the total soluble solids (%Brix) of the hydrolysate ($p < 0.05$). During the early fermentation stage (days 2 to 3), treatment combinations containing higher glucoamylase proportions exhibited significantly higher total sugar contents compared to the low glucoamylase ratio. This is due to the synergistic and rate-limiting mechanism of glucoamylase during saccharification. While α -amylase acts as an endo-enzyme that randomly cleaves internal α -1,4-glucosidic bonds in starch to produce short-chain dextrans and oligosaccharides, it cannot efficiently liberate free glucose molecules (Liu et al., 2026). In contrast, glucoamylase functions as an exo-enzyme that sequentially hydrolyzes both α -1,4 and α -1,6 glucosidic linkages from the non-reducing ends of dextrans to produce fermentable D-glucose (Mendonça et al., 2023). Higher glucoamylase concentrations accelerate the rate-limiting step of saccharification, resulting in rapid accumulation of fermentable sugars in the initial phase despite ongoing microbial consumption. Conversely, restricting glucoamylase to 0.5% limits glucose liberation, producing a lower Brix yield.

By Day 4, a general decline in sugar content was observed across all enzyme treatments. The treatment with high α -amylase and low glucoamylase (1.5% : 0.5%) dropped to its lowest recorded value ($10.00 \pm 0.00\%$ brix), while the 0.5% : 1.5% ratio maintained a relatively high concentration ($13.00 \pm 1.41\%$ Brix). This is due to between Days 3 and 4, the rate of carbon source consumption by fermenting microorganisms (which metabolize simple sugars into secondary metabolites such as ethanol) exceeds the rate of enzymatic sugar generation. In the 1.5% : 0.5% ratio, the low concentration of glucoamylase becomes fully exhausted or substrate-limited, failing to replenish glucose as fast as the microbes consume it. In contrast, higher glucoamylase dosages provide sustained enzymatic saccharification that partially offsets microbial uptake.

The alcohol content (%)

The alcohol content is shown in Table 2.

Table 2. The alcohol content (%)

Fermentation time (days)	α -Amilase : Glukoamilase			
	(0.5 : 1.5)%	(1 : 1)%	(1.5 : 0.5)%	Average
2	46.00 ± 1.41^d	10.00 ± 0.00^a	5.00 ± 0.00^a	20.33 ± 20.01^q
3	31.00 ± 1.41^c	9.50 ± 0.70^a	32.50 ± 3.53^c	24.33 ± 11.63^r
4	6.00 ± 1.41^a	35.00 ± 7.07^c	20.00 ± 0.00^b	20.33 ± 13.36^q
5	5.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^p
Average	22.00 ± 18.55^y	14.88 ± 12.87^x	15.63 ± 12.37^x	

Note: Means within columns or rows followed by different letters indicate significant differences based on Duncan's Multiple Range Test at the 5% significance level.

The ratio treatment of α -amylase to glucoamylase significantly affected the alcohol content of the bioethanol produced. Treatment A1 (0.5% : 1.5%) yielded the highest average alcohol content at 22.00%. Peak alcohol production was achieved at different fermentation durations across treatments: A1 reached its peak on day 2 (B1; 46%), A3 on day 3 (B2; 32.5%), and A2 on day 4 (B3; 35%). This variation occurred because the enzyme ratio dictates the rate of glucose supply for the yeast, rather than the total amount of available glucose.

As greater amounts of sugar were converted into ethanol, the alcohol concentration correspondingly increased. However, prolonged fermentation led to nutrient depletion and ethanol accumulation, which inhibited yeast activity and ultimately decreased alcohol yield. These findings align with Wang et al., (2021), who reported that successful ethanol production depends not only on the fermentative capacity of the yeast, but also on the availability of fermentable sugars generated during the saccharification stage.

The treatment ratio of 0.5% α -amylase to 1.5% glucoamylase achieved the highest overall alcohol content ($46.00 \pm 1.41\%$) as early as Day 2. Beyond Day 2, ethanol levels significantly declined to 31.00% (Day 3), 6.00% (Day 4), and reached a baseline of 5.00% by Day 5. Glucoamylase acts as the rate-limiting enzyme in glucose liberation by cleaving both α -1,4 and α -1,6-glucosidic bonds from the non-reducing ends of liquefied starch (Shadbahr et al., 2017). A high concentration of glucoamylase (typically up to 1.5%) rapidly converts available dextrins into monomeric D-glucose, driving fast glycolysis and accelerated ethanol synthesis by fermenting yeasts during early incubation (Tsfaye et al., 2025). However, once substrate depletion occurs, extended incubation under micro-aerobic environments facilitates the subsequent oxidation of ethanol into acetic acid or ethyl acetate by secondary microflora, resulting in a sharp drop in overall alcohol content (Ge, 2025). Excessive α -amylase accelerates endo-cleavage, yielding abundant short-chain dextrins. However, an insufficient glucoamylase proportion (such as 0.5%) acts as a severe kinetic bottleneck that restricts the final rate of glucose release (Bunga et al., 2025). This limitation delays peak ethanol generation—shifting it from Day 2 to Day 3 under suboptimal ratios—and results in a significantly lower maximum ethanol yield due to the incomplete saccharification of complex oligosaccharides.

The density of bioethanol (g/mL)

The density of bioethanol is shown in Table 3.

Table 3. The density of bioethanol (g/mL)

Fermentation time (days)	α -Amilase : Glukoamilase			Average
	(0.5 : 1.5)%	(1 : 1)%	(1.5 : 0.5)%	
2	0.94 ± 0.00^a	0.99 ± 0.00^d	0.99 ± 0.00^d	0.97 ± 0.02^q
3	0.96 ± 0.00^b	0.99 ± 0.00^d	0.96 ± 0.66^b	0.97 ± 0.01^p
4	0.99 ± 0.00^d	0.95 ± 0.00^b	0.97 ± 0.00^c	0.97 ± 0.01^p
5	0.99 ± 0.00^d	0.99 ± 0.00^d	0.99 ± 0.00^d	
Average	0.97 ± 0.02^x	0.98 ± 0.01^z	0.97 ± 0.01^y	

Note: Means within columns or rows followed by different letters indicate significant differences based on Duncan's Multiple Range Test at the 5% significance level.

The ratios of 1% : 1% exhibited the significantly highest mean density of 0.98 ± 0.01 g/mL, whereas treatments (ratio of 0.5% : 1.5% and 1.5% : 0.5%) recorded densities of 0.97 ± 0.02 g/mL and 0.97 ± 0.01 g/mL, respectively (Table 3). These empirical findings corroborate the analysis of variance (ANOVA), which revealed a highly significant interaction effect ($p < 0.01$) between the α -amylase-to-glucoamylase ratio and fermentation duration on bioethanol density. This indicates that the density response to variations in the enzymatic ratio is strongly dependent on the fermentation time.

Furthermore, extended fermentation periods facilitated greater bioconversion of sugars into ethanol, thereby inducing a progressive decrease in the solution density. The prolonged incubation heightened microbial metabolic activity, leading to extensive carbohydrate degradation. Because ethanol possesses a lower specific gravity than water, the increased

volumetric fraction of alcohol within the binary mixture systematically reduced the overall liquid density (Visca et al., 2020).

These observed variations suggest that modulating the α -amylase to glucoamylase ratio elicits differential density profiles. This phenomenon is primarily attributable to the disparate catalytic efficiencies of the enzyme combinations in depolymerizing starch into fermentable monosaccharides. Ultimate bioethanol density is governed by the ethanol yield, residual moisture content, and the presence of volatile impurities in the distillate (Cripwell et al., 2020). Consequently, optimizing the synergistic enzyme ratio is critical for tailoring the physicochemical properties of the resulting bioethanol.

The two-way Analysis of Variance (ANOVA) showed a significant interaction effect ($p < 0.05$) between the enzyme concentration ratio (α -amylase : glucoamylase) and fermentation duration on the density of the bioethanol hydrolysate (Table 3). Because pure ethanol has a significantly lower density (0.789 g/mL at 20 °C) than water (1.00 g/mL), the measured density serves as an inverse physical indicator of alcohol concentration—where lower density values correspond directly to higher ethanol accumulation in the mixture. The treatment combination of 0.5% α -amylase and 1.5% glucoamylase achieved the lowest overall density (0.94 ± 0.00 g/mL) on Day 2. As fermentation progressed to Day 3, the density increased to 0.96 ± 0.00 g/mL, and eventually reached 0.99 ± 0.00 g/mL on Days 4 and 5. This sharp drop in density on Day 2 perfectly correlates with the peak alcohol content (46.00%) observed in Table 2. The rapid enzymatic conversion of starch into glucose by the elevated glucoamylase level provided immediate substrate for yeast glycolysis, leading to rapid ethanol production that significantly lowered the specific gravity of the liquid matrix.

The pH of bioethanol (g/mL)

The pH of bioethanol is shown in Table 4.

Table 4. The pH of bioethanol (g/mL)

Fermentation time (days)	α -Amilase : Glukoamilase			
	(0.5 : 1.5)%	(1 : 1)%	(1.5 : 0.5)%	Average
2	6.55 ± 0.07^f	4.18 ± 0.00^b	4.06 ± 0.00^a	4.93 ± 1.25^q
3	6.15 ± 0.03^d	4.20 ± 0.01^b	6.24 ± 0.0^{dc}	5.53 ± 1.03^s
4	4.09 ± 0.02^{ab}	6.29 ± 0.13^c	5.30 ± 0.00^c	5.23 ± 0.98^r
5	4.05 ± 0.00^a	4.09 ± 0.01^{ab}	4.05 ± 0.00^a	
Average	5.21 ± 1.22^z	4.69 ± 0.99^x	4.91 ± 0.98^y	

Note: Means within columns or rows followed by different letters indicate significant differences based on Duncan's Multiple Range Test at the 5% significance level.

According to Table 4, the treatment combination A1B1 (α -amylase to glucoamylase ratio of 0.5% : 1.5% with a 2-day fermentation duration) yielded the highest pH value of 6.55, whereas combinations A1B4 and A3B4 exhibited the lowest pH value of 4.05. These results indicate that the synergistic interaction between the enzyme ratio and fermentation period exerts a differential effect on the pH profile of the post-distillation bioethanol.

The higher enzymatic proportion paired with a short fermentation timeframe is posited to produce bioethanol with lower acidity relative to other treatment parameters. Conversely, the marked decline in pH observed during the 5-day fermentation treatments demonstrates that prolonged incubation induces substantial alterations in the chemical characteristics of the bioethanol, resulting in heightened acidity. This trend aligns with Ruhibnur et al., (2019), who reported that varying fermentation durations yield distinct bioethanol characteristics, with pH serving as a key indicator of product acidity. Consequently, fermentation duration governs not only the total ethanol yield but also fundamentally dictates the chemical properties of the final bioethanol product (Siregar et al., 2024).

The two-way ANOVA indicated a significant interaction effect ($p < 0.05$) between the enzyme concentration ratio (α -amylase : glucoamylase) and fermentation duration on bioethanol pH (Table 4). The overall shift toward acidity (pH~4.0) across treatments is driven

by yeast organic acid production, whereas transient pH increases coincide with peak ethanol accumulation.

Water content (%)

The water content was calculated based on alcohol content measurements using an alcoholmeter by subtracting the alcohol content from 100%. Estimation of the water content is shown in Table 5.

Table 5. Water content (%)

Fermentation time (days)	α -Amilase : Glukoamilase			
	(0.5 : 1.5)%	(1 : 1)%	(1.5 : 0.5)%	Average
2	54,00 $\pm$ 1,41 ^a	88,00 $\pm$ 2,82 ^c	95,00 $\pm$ 0,00 ^f	79,00 $\pm$ 19,66 ^q
3	69,00 $\pm$ 1,41 ^c	90,50 $\pm$ 0,70 ^e	67,50 $\pm$ 3,53 ^c	75,67 $\pm$ 11,63 ^p
4	94,00 $\pm$ 1,41 ^f	65,00 $\pm$ 1,41 ^b	80,00 $\pm$ 0,00 ^d	78,33 $\pm$ 14,84 ^q
5	95,00 $\pm$ 0,00 ^f	95,00 $\pm$ 0,00 ^f	95,00 $\pm$ 0,00 ^f	
Average	78,00 $\pm$ 18,55 ^x	83,63 $\pm$ 14,27 ^y	84,37 $\pm$ 12,37 ^y	

Note: Means within columns or rows followed by different letters indicate significant differences based on Duncan's Multiple Range Test at the 5% significance level.

Table 5 confirms that treatment A1 achieved the significantly lowest mean moisture content at 78.00%, which was statistically distinct from A2 (83.63%) and A3 (84.37%). Regarding Factor B (fermentation duration), a 3-day fermentation period (B2) yielded the lowest moisture content (75.67%), followed by B1 (79.00%) and B3 (78.33%), while B4 recorded the significantly highest moisture content at 95.00%. This trend exhibits an inverse relationship with the alcohol concentration profile, thereby validating the complementary relationship between alcohol and moisture content, expressed as moisture content (%) = 100% – alcohol content (%) according to the analytical procedure adapted from Sulaiman et al., (2024)

As presented in Table 5, the moisture content during starch-based fermentation represents a complex function governed by three interdependent mechanisms: the rates of enzymatic hydrolysis, substrate consumption, and ethanol synthesis. These parameters interact in a non-linear manner. Alterations in bioethanol moisture content are predominantly driven by the synergistic interaction between the enzyme ratio and fermentation duration rather than by either factor independently. In other words, the effect of the enzymatic ratio on moisture content varies dynamically as a function of incubation time (Jannah et al., 2024).

The low moisture content observed in treatment A1 indicates that a higher proportion of glucoamylase enhances the catalytic depolymerization of starch into glucose, thereby increasing substrate availability for *Saccharomyces cerevisiae*. This condition facilitates a more efficient metabolic conversion into ethanol, leaving a reduced residual water fraction in the final product. These findings closely align with Soeprijanto et al., (2022), who reported that effective synergy between α -amylase and glucoamylase activity optimizes starch breakdown, promoting higher fermentation efficiency and elevated bioethanol yields.

CONCLUSION

The α -amylase-to-glucoamylase ratio significantly affected total sugar content, alcohol concentration, moisture content, pH, and density of the bioethanol. Additionally, fermentation duration had a highly significant effect on alcohol concentration, moisture content, pH, and density, while showing no significant effect on total sugar content. Considering overall bioethanol quality—prioritizing maximum alcohol yield (46.00%) alongside favorable density, pH, and moisture content—the treatment combining a 0.5% α -amylase to 1.5% glucoamylase ratio with a 2-day fermentation duration was determined to be the best condition.

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