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Optimized bioethanol production from banana stem waste via simultaneous saccharification and fermentation with *Saccharomyces cerevisiae*

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ABSTRACT Indonesia, one of the world's largest banana producers, generates significant quantities of banana stem waste, leading to environmental challenges. This study explores the potential of converting this lignocellulosic biomass into bioethanol using a combination of steam pretreatment and simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae*. The SSF process integrates enzymatic hydrolysis and fermentation, streamlining bioethanol production. The research applied the Taguchi method with an $L_9(3^4)$ orthogonal array to optimize key parameters, including enzyme concentration, particle size, temperature, and pH. Optimal conditions—5% enzyme concentration (v/v), 60 mesh banana powder, 35 °C and pH 5.00—yielded a maximum ethanol concentration of 9 g/L. Enzyme concentration and particle size were identified as critical factors in enhancing bioethanol yield. This study highlights the potential of banana stem waste as a sustainable resource for bioethanol production, contributing to waste reduction and renewable energy development.

KEYWORDS Banana stem waste; Bioethanol; Bioprocess optimization; Lignocellulosic biomass; Simultaneous saccharification and fermentation (SSF)

1. Introduction

Indonesia is one of the largest banana-producing countries globally, generating substantial quantities of banana stem waste as a by-product of cultivation. While the fruit itself holds economic value, the stem is often discarded, creating significant environmental concerns. Banana stem waste represents a significant biomass source in Indonesia and contains substantial amounts of lignocellulose: 46% cellulose, 9% lignin, and 38.54% hemicellulose (Suryaningsih and Pasaribu 2015; Guerrero et al. 2018; Sawarkar et al. 2022). The high cellulose content positions it as a promising raw material for bioethanol production. Utilizing this waste for bioethanol production simultaneously addresses two critical issues: reducing agricultural waste and providing a renewable energy source.

Bioethanol is a widely recognized biofuel with the potential to reduce dependence on fossil fuels and mitigate greenhouse gas emissions (Bušić et al. 2018; El-Araby 2024). The pretreatment of lignocellulosic biomass is critical for efficient bioethanol production. The primary goal of pretreatment is to modify the structure of lignocellulose, making cellulose more accessible to the enzymes responsible for breaking down saccharide polymers into sugar monomers. This accessibility facilitates higher yields of glucose and xylose (Zeghlouli et al. 2021), ultimately enhancing the enzymatic conversion of cellulose.

The saccharification process involves the hydrolysis or breakdown of cellulose into simple sugars. This is achieved through the action of endoglucanase, exoglucanase, and β-glucosidase enzymes, which are part of the cellulase enzyme group. These enzymes work synergistically to degrade cellulose into glucose, facilitating the production of reducing sugars at high concentrations (Ingale et al. 2014; Kusmiyati et al. 2018). Additionally, xylanase enzymes play a crucial role in degrading hemicellulose into xylo-oligosaccharides and xylose monomers.

Fermentable sugars resulting from the hydrolysis and saccharification stages, including glucose, fructose, and sucrose, can be utilized by *Saccharomyces cerevisiae* to produce ethanol. However, the efficiency of fermentation varies depending on the sugar composition, as *S. cerevisiae* preferentially consumes glucose over fructose, often leaving residual fructose in the medium at the end of fermentation. This phenomenon, known as glucose-fructose discrepancy, has been attributed to differences in sugar transport mechanisms and phosphorylation kinetics within yeast cells (Jasman et al. 2015).

Bioethanol production can be conducted using two primary methods: the simultaneous saccharification and fermentation (SSF) and the separate hydrolysis and fermen-

tation (SHF) processes. The SSF process offers several advantages over the SHF method, including faster hydrolysis, reduced enzyme requirements, higher product yield, less need for sterile conditions as glucose is directly converted to ethanol, and shorter overall process time (Kusmiyati et al. 2018).

In the production of bioethanol from banana stem waste, the SSF technology has demonstrated significant efficacy. The highest recorded bioethanol concentration using SSF was 8.51 g/L at a pH of 5.00, utilizing an enzyme mix from *Aspergillus niger*, *Trichoderma reesei*, and *Zymomonas mobilis* in a 1:1:2 ratio (Kusmiyati et al. 2018). Other studies have shown that using banana pseudo-stem raw materials with SSF resulted in a bioethanol concentration of 4.32 g/L (Kusmiyati et al. 2018), while banana kapok pseudo-stem processed through SSF achieved a concentration of 0.05% (v/v) (Sulfiani et al. 2019). In contrast, under different experimental conditions, the SHF method produced yielded 4.20 g/L of bioethanol (Adeniji et al. 2010).

This study addresses the under-utilization of banana stem waste by demonstrating its potential as a sustainable feedstock for bioethanol production. The research focuses on optimizing SSF conditions to maximize yield while addressing the challenges of enzymatic hydrolysis and fermentation. Key process variables include enzyme concentration, banana stem powder particle size, temperature, and pH. The physical and enzymatic pretreatment stages are designed to efficiently degrade lignocellulose and hemicellulose into cellulose. The Taguchi method with an $L_9(3^4)$ orthogonal array design, systematically investigates key process parameter to identify optimal conditions and critical factors influencing bioethanol yield. This research not only highlights the potential of banana stem waste as a sustainable bioresource but also contributes to advancements in renewable energy technologies.

2. Materials and Methods

2.1. Banana powder processing

The method for preparing banana powder was modified from Idrees et al. (2013). The banana stems were dried in a convection oven at 65 °C for 48 h until moisture content was reduced to below 10%, followed by grinding using a high-capacity Waring blender. The powder was sieved into three categories: 40 mesh (0.420 mm), 50 mesh (0.297 mm), and 60 mesh (0.250 mm). This process was crucial for reducing the particle size, which facilitates more efficient hydrolysis by increasing the surface area available for enzymatic action.

2.2. Sieving banana stem powder

Banana stem powder sieving was conducted using a CBN brand sieve test tool. Three different sieve pore sizes were used: 40 mesh banana powder, 50 mesh banana powder, and 60 mesh banana powder. The objective of this process was to standardize the particle size of the banana stem powder to ensure uniformity. Reducing particle size, particularly for hemicellulose and cellulose, enhances the interaction between these compounds and the enzymes. This improvement in enzyme-substrate affinity facilitates a more efficient conversion of cellulose into glucose, thereby optimizing the hydrolysis step of the bioethanol production process (Khienpanya et al. 2015).

2.3. Proximate analysis

Proximate analysis was performed to quantitatively assess various components of the banana stem biomass. This analysis involved measuring the fixed carbon content, volatile matter, moisture content, ash concentration, nitrogen concentration, protein concentration, and fat concentration. The fixed carbon content, volatile matter, moisture content, and ash concentration were determined according to the ASTM D4442-16 standard (D07 Committee 2015; E48 Committee 2011). Initially, the moisture content (MC) was measured using the oven-drying method, where samples were dried in a forced-convection oven maintained at 103 ± 2 °C until a constant weight was achieved. The MC was calculated using the formula:

$$MC\ \% = \frac{A - B}{B} \times 100\% \quad (1)$$

where A represents the initial mass of the sample (g) and B the oven-dry mass (g). The ash content was determined by combusting the oven-dried sample in a muffle furnace at approximately 600 °C until only inorganic residue remained. The volatile matter content was measured by heating the oven-dried sample in a closed crucible at 950 °C, and the resulting weight loss, excluding moisture, was recorded. The fixed carbon content was calculated indirectly as the remaining percentage after accounting for moisture, volatile matter, and ash content using the formula:

Fixed Carbon content (%)

$$= 100 - (MC + Volatile\ Matter + Ash\ Content) \quad (2)$$

All measurements were conducted in triplicate to ensure accuracy and reproducibility, and results were reported as percentages relative to the oven-dry weight of the sample.

The nitrogen and protein content of the banana stem powder samples were determined using the Kjeldahl method, following ISO 5983-1:2005 standards. In this procedure, organic matter in the sample is digested with concentrated sulfuric acid in the presence of a catalyst (potassium sulfate and copper(II) sulfate) to convert nitrogen into ammonium sulfate. After digestion, the mixture is rendered alkaline with sodium hydroxide, liberating ammonia. The ammonia is distilled and absorbed into a boric acid solution, where it is titrated with a standardized sulfuric acid solution. The nitrogen content (N) is calculated based on the volume of titrant used, the molar mass of nitrogen, and the sample mass. Crude protein content is estimated by multiplying the nitrogen content by a

conventional factor (N×6.25), assuming all nitrogen originates from protein. The method was performed in triplicate to ensure accuracy, and results were reported to the nearest 0.01 g/kg.

Crude lipid content was determined using the Soxhlet extraction method, following AOAC 2003.05 guidelines. Approximately 100 g of the finely powdered sample (sieved to 60 mesh) was weighed and wrapped in filter paper. The Soxhlet apparatus, connected to a heating mantle, was assembled, and the sample was placed in the extraction chamber. Hexane (150 mL, pro analysis grade) was used as the lipid solvent. The system was operated for 1.5 reflux cycles to ensure complete lipid extraction.

After extraction, the lipid-rich hexane was separated using a rotary evaporator to remove the solvent. The remaining lipid residue was dried in an oven at 105 °C for 1 h to remove any residual solvent. Lipid content was determined gravimetrically by weighing the lipid residue. The percentage of lipid content was calculated using the following formula:

$$Lipid\ content\ (\%) = \big((Weight\ of\ flask\ with\ lipid\ (g) - Weight\ of\ empty\ flask\ (g))/Sample\ weight\ (g)\big) \times 10) \quad (3)$$

2.4. Cellulose content measurement

The measurement of cellulose content in banana stem powder was conducted using the Chesson-Datta method, proposed by Chesson (1988). This method is designed to quantitatively calculate the lignocellulose content, which includes cellulose, hemicellulose, and lignin. Initially, 1 g of banana stem powder (a) was refluxed with 120 mL of distilled water at 100 °C for one hour. After refluxing, the sample was filtered and dried, and the residue was weighed (b). This residue was then subjected to further refluxing with 150 mL of 0.5 M sulfuric acid (H_2SO_4) solution for one hour, then filtered, dried, and weighed (c). Following this, the residue underwent an additional treatment, soaked with 10 mL of 72% H_2SO_4 solution for four hours, then filtered, dried, and weighed (d). The final residue was dried once more and weighed (e). Using these weights (g), the percentage of each chemical component was calculated according to the Chesson-Datta analytical method, which helps in understanding the proportion of cellulose, hemicellulose, and lignin in the biomass.

$$Hot\ water\ soluble\ \% = \frac{a-b}{a} \times 100\% \quad (4)$$

$$Hemicellulose\% = \frac{b-c}{a} \times 100\% \quad (5)$$

$$Cellulose\% = \frac{c-d}{a} \times 100\% \quad (6)$$

$$Lignin\% = \frac{d-e}{a} \times 100\% \quad (7)$$

$$Ash\% = \frac{e}{a} \times 100\% \quad (8)$$

2.5. Saccharomyces cerevisiae culture preparation

Saccharomyces cerevisiae, obtained from the Institut Teknologi Bandung culture collection (School of Life Science and Technology), was the yeast used in the fermentation process and required careful preparation to ensure optimal activity. Prior to fermentation, the yeast undergoes subculture and adaptation through three activation stages to enhance its fermentative capacity. The growth medium used for these stages includes potato dextrose agar (PDA) and potato dextrose broth (PDB). The PDA medium is composed of 40 g/L of potato extract, 10 g/L of glucose, and 15 g/L of agar. Meanwhile, the PDB medium consists of 20 g/L of potato extract and 6.5 g/L of glucose. Both media are sterilized in an autoclave at 121 °C under 1.5 atm of pressure to eliminate any potential contaminants. This preparatory step is crucial for acclimatizing the yeast to the growth conditions and ensuring a robust start to the fermentation process.

2.6. Enzyme preparation

The enzymatic conversion process was carefully evaluated by preparing and determining the concentration of cellulase and xylanase enzymes, using modified methods of Zeghlouli et al. (2021). These enzymes were used at varying concentrations to optimize their activity in the hydrolysis process. The concentrations tested were 1% v/v (10 UI), 2.5% v/v (25 UI), and 5% v/v (50 UI). Specifically, the cellulase enzyme utilized was Viscozyme® Cassava CL from Novozymes, which exhibits an enzymatic activity of 700 EGU/g. For the breakdown of hemicellulose, the endo-1,4-β-Xylanase from Megazyme® (190 U/mg) was used. These concentrations and enzyme choices were strategically selected to effectively catalyze the conversion of complex polysaccharides in the biomass into simpler, fermentable sugars, essential for efficient bioethanol production.

2.7. Simultaneous saccharification and fermentation (SSF) optimization process

SSF process integrates enzyme hydrolysis and fermentation within the same reactor, a 250 mL flask, to enhance the efficiency and speed of bioethanol production (Olofsson et al. 2008). This process utilizes cellulase and xylanase enzymes alongside *Saccharomyces cerevisiae*, a yeast known for its fermentative capabilities. Prior to initiation of the SSF, all process tools, including the flask, must be sterilized at a temperature of 121 °C and a pressure of 1.5 atm for 15 min in an autoclave to ensure a sterile environment (Kusmiyati et al. 2018)

The SSF medium, prepared in a total volume of 150 mL, contained 6 g of banana powder, 0.15 g of $(NH_4)_2SO_4$, 0.075 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of urea, and deionized water to make up the final volume with *S. cerevisiae* inoculum concentration of 10% (v/v) at a density of 10^6 cells/mL. The enzymatic concentration includes cellulase and xylanase derived from the optimization results of enzyme hydrolysis, available in concentrations of 1%, 2.5%, and 5%

(v/v). This formulation also considers various sieve pore sizes of the banana stem substrate (40 mesh, 50 mesh, 60 mesh), and operates under a range of temperatures (30 °C, 35 °C, 40 °C) and pH values (5.00, 6.00, 7.00) to find the optimal conditions for maximum bioethanol yield. Control experiments were conducted without enzyme addition and steam pretreatment to benchmark these results against the optimized conditions.

2.8. Cell quantification

Calculating cell numbers provides insight into the growth kinetics of Saccharomyces cerevisiae during SSF and its correlation with ethanol production. Cell density was determined using a hemocytometer to ensure precise quantification of the yeast population (Gilliland 1959). Initially, 100 µL of the sample inoculum was diluted with 9 mL of 0.85% physiological NaCl solution, achieving a suitable dilution for analysis. This diluted sample was carefully loaded onto the surface of the hemocytometer to completely cover the grid area of the counting chamber. For accurate differentiation between live and dead cells, the cells were stained using methylene violet stain at a concentration of 0.01% (w/v) in a 2% sodium citrate solution. The hemocytometer was then placed under a microscope set to 400× magnification, allowing for detailed observation and counting of the stained cells. This method provides a reliable measure of cell viability and concentration necessary for optimizing fermentation conditions.

2.9. Reducing sugar and ethanol measurement

Reducing sugar and ethanol concentration was measured using HPLC, which is well-suited for sugar and bioethanol analysis. GC-MS was not employed due to resource constraints and the adequacy of HPLC for the study objectives The HPLC method was used for reducing sugar and ethanol measurement in this study was performed using a Shimadzu Prominence SPD-20A system, equipped with a VWD - Double W detector and an SCR 101-C column. For this study, 100 mg of the sample was dissolved in 10 mL of deionized water to achieve a 1% (w/v) solution. The prepared solution was centrifuged at 6000 rpm for 15 min at 4 °C to remove insoluble debris. The supernatant was then filtered through a 0.2 µm cellulose acetate syringe filter to ensure removal of any remaining particulates. Finally, the filtered solution was transferred into a 1 mL autosampler vial, ensuring it was free of contaminants and ready for injection into the HPLC system. The operational parameters included a mobile phase consisting of a deionized water solution, with a flow rate set at 1 mL/min, and the column oven temperature maintained at 80 °C. An injection volume of 20 µL was used for each sample. The selection of the mobile phase composition was meticulously optimized to achieve the best separation efficiency, which was determined based on retention time (tR) and peak area analysis. This HPLC setup was crucial for accurately measuring the concentrations of various types of reducing sugars, such as glucose and fructose, as well as bioethanol, throughout the banana stem simultaneous saccharification and fermentation (SSF) process.

2.10. Measurement of protein and ammonium content in fermentation broth

The concentration of protein in the fermentation broth was determined using the Bradford assay (Kielkopf et al. 2020). For this procedure, 0.1 mL of the sample solution was mixed with 5 mL of Bradford reagent. The mixture was thoroughly homogenized using a vortex mixer to ensure uniformity. The absorbance of the resultant solution was then measured using a UV-Vis 900 spectrophotometer set at a wavelength of 595 nm.

The ammonium concentration in the banana stem solution was assessed using the Nessler method (Jeong et al. 2013). Initially, 0.1 mL each of Seignette reagent and Nessler reagent were added to 5 mL of the sample solution. The mixture was then agitated using a vortex mixer to ensure thorough mixing. After allowing the solution to stand for ten minutes to react fully, the absorbance was measured at 420 nm using a spectrophotometer. The ammonium content was subsequently interpolated from a standard curve established during the experiment, providing a precise quantification of ammonium levels in the sample. The nitrogen content analysis provides a general overview of protein content in the fermentation broth, while the crude protein content assay is used to measure soluble protein concentrations in fermentation media, crucial for understanding nutrient availability.

2.11. Data analysis

The research employed the Taguchi experimental design method (Taguchi 1960), utilizing an orthogonal array $L_9(3^4)$ to systematically analyze the effects of multiple variables on bioethanol production (Azmi et al. 2011). This method was implemented using Minitab 21 software, a statistical tool chosen for its robust data analysis capabilities. The primary aim of adopting the Taguchi method in this study was to optimize the production process by identifying the most influential factors before actual production begins, serving as an effective form of offline quality control. This approach not only enhances the efficiency and yield of the production process but also minimizes variability and improves overall product quality by systematically varying process parameters and analyzing their impact.

2.12. Orthogonal array

Orthogonal arrays are a fundamental component of the Taguchi method due to their unique structure where, for each level of a factor, the sum of all levels is equal. This consistency ensures that the influence of one factor on experiment outcomes is isolated from other factors. As a result, researchers can more easily determine which factors most significantly impact the process outcomes. Orthogonal arrays facilitate the systematic control and adjustment of variable levels, enhancing the efficiency and accuracy of the experimental design (Table 1). These arrays are represented in the experimental layout by the equation

TABLE 1 Taguchi experimental design with Orthogonal array $L_9(3^4)$

Trial	Factor			
	A	B	C	D
1	1%	40 mesh	30 °C	5
2	1%	50 mesh	35 °C	6
3	1%	60 mesh	40 °C	7
4	2.50%	40 mesh	35 °C	7
5	2.50%	50 mesh	40 °C	5
6	2.50%	60 mesh	30 °C	6
7	5%	40 mesh	40 °C	6
8	5%	50 mesh	30 °C	7
9	5%	60 mesh	35 °C	5

(9), which defines the configuration and interactions of the variables involved. This structured approach ensures precise experimental control, ensuring that the data collected is both reliable and robust, ideal for optimizing production processes.

$$Ln(l^f) \tag{9}$$

With:
L: orthogonal array,
n: number of rows/trials,
l: number of levels,
f: number of factors.

The signal-to-noise ratio (SNR) is a critical metric in quality control and plays a pivotal role in the Taguchi method. It is defined as the logarithm of a quadratic loss function and is used to quantitatively assess the quality of a product. In the context of optimizing production processes, SNR helps determine the robustness of process parameters against variability. For the ”larger is the better” quality category, which is often applied in scenarios where the objective is to maximize a response variable, the equation used to calculate SNR (equation 10) emphasizes that a higher SNR value indicates better quality. This approach allows researchers and engineers to identify and implement process settings that enhance product performance by maximizing the SNR (Azmi et al. 2011).

$$SNR = -10log10(\frac{1}{r}\sum_{i=1}^{r}\frac{1}{(yi)^2}) \tag{10}$$

3. Results and Discussion

3.1. Results

The simultaneous saccharification and fermentation (SSF) process plays a crucial role in maximizing bioethanol production, with several key factors influencing its efficiency. These factors include the concentration of enzymes (expressed as %, v/v), the sieve pore size of the biomass (mesh), the operational temperature, and the pH of the medium. Optimal conditions were determined to be an enzyme concentration of 5% (v/v), 60 mesh banana powder, a temperature of 35 °C, and a pH of 5.00. Under these conditions, the highest bioethanol yield reached 9.0 g/L, as recorded in Table 2.

In the control setup devoid of enzymes, the maximum bioethanol yield was 5.0 g/L (Table 3). However, when steam pretreatment was included without enzymes, the yield improved to 7.2 g/L (Table 4). This indicates that even in the absence of enzymatic action, the physical pre-

TABLE 2 Optimization factors on bioethanol production during SSF process using enzymes and steam treatment.

Trial	Bioethanol production 1 (g/L)	Bioethanol production 2 (g/L)	Average	SNR
1	7.2	7.1	7.15	17.0855
2	7.3	7.3	7.30	17.2665
3	7.4	7.6	7.50	17.4989
4	7.5	7.6	7.55	17.5584
5	7.7	7.8	7.75	17.7855
6	8.0	7.9	7.95	18.0068
7	8.3	8.4	8.35	18.4333
8	8.5	8.7	8.60	18.6882
9	9.0	9.0	9.00	19.0849

TABLE 3 Optimization factors on bioethanol production during SSF process without enzyme.

Trial	Bioethanol production 1 (g/L)	Bioethanol production 2 (g/L)	Average	SNR
1	4.0	4.0	4.00	12.0412
2	4.1	4.1	4.10	12.2557
3	4.0	4.1	4.05	12.1471
4	4.3	4.2	4.25	12.566
5	4.4	4.4	4.40	12.8691
6	4.5	4.5	4.50	13.0643
7	4.7	4.8	4.75	13.5324
8	5.0	5.0	5.00	13.9794
9	4.9	4.9	4.90	13.8039

TABLE 4 Optimization factors on bioethanol production during SSF process without steam treatment.

Trial	Bioethanol production 1 (g/L)	Bioethanol production 2 (g/L)	Average	SNR
1	6.1	6.1	6.10	15.7066
2	6.2	6.2	6.20	15.8478
3	6.2	6.3	6.25	15.9168
4	6.5	6.5	6.50	16.2583
5	6.6	6.6	6.60	16.3909
6	6.7	6.7	6.70	16.5215
7	6.9	6.8	6.85	16.7131
8	7.0	7.0	7.00	16.9020
9	7.2	7.2	7.20	17.1466

treatment enhances the substrate's accessibility, albeit less efficiently than when enzymes are present. The significant increase in yield under optimized conditions can be attributed to the synergistic effects of cellulase and xylanase enzymes, which effectively convert cellulose into glucose, thereby optimizing the sugar availability for fermentation by *Saccharomyces cerevisiae* cells.

The signal-to-noise ratio (SNR) values, essential for evaluating the quality and robustness of the bioethanol production process, were analyzed across several factors and levels under the "larger is the better" category (Azmi et al. 2011). The highest SNR values were observed under the optimal conditions of 5% enzyme concentration (v/v), 60 mesh banana stem powder, 35 °C temperature, and pH 5.00, as shown in Figure 1a. These conditions proved most effective for maximizing bioethanol yield.

Further analysis was conducted to assess the impact of removing certain elements from the process. When enzymes were excluded, the highest SNR values were still prominent with a 60-mesh sieve pore size, a temperature of 35 °C, and a pH of 5.00, indicating that these factors independently contribute significantly to the process efficacy (Figure 1b). Additionally, excluding steam treatment revealed that the combination of 5% enzyme concentration (v/v), 60 mesh banana stem powder, 35 °C temperature, and pH 5.00 continued to yield the highest SNR values (Figure 1c). This suggests that even without steam pretreatment, the selected enzyme concentration and process conditions effectively enhance bioethanol production.

The growth kinetics of *Saccharomyces cerevisiae* and bioethanol production (Figure 3) were significantly influenced by the presence of enzymes and steam treatment. Under optimized conditions (with enzymes and steam treatment), yeast biomass showed rapid growth, entering the exponential phase within 18 hours and achieving a maximum ethanol yield of 9.0 g/L. In contrast, the absence of enzymes or steam treatment delayed yeast growth and reduced ethanol production, with yields of 5.0 g/L and lower growth rates observed. Protein and ammonium concentrations in the fermentation broth (Figure 4 and Figure 5) followed a similar trend, with both decreasing significantly during the first 24 hours as *S. cerevisiae* utilized them as nitrogen sources for growth and metabolic activity. This utilization directly correlated with increased ethanol production, stabilizing after 24 hours when nitrogen demand lessened.

The pH profile during the fermentation process (Figure 6) also played a critical role, with an optimal range of 4.41–5.00 supporting maximum ethanol production. Maintaining this range enhanced yeast metabolic activity and fermentation efficiency, while deviations negatively affected bioethanol yield. Collectively, these results highlight the importance of enzymes, steam treatment, nitrogen availability, and pH control in maximizing bioethanol production during the simultaneous saccharification and fermentation (SSF) process.

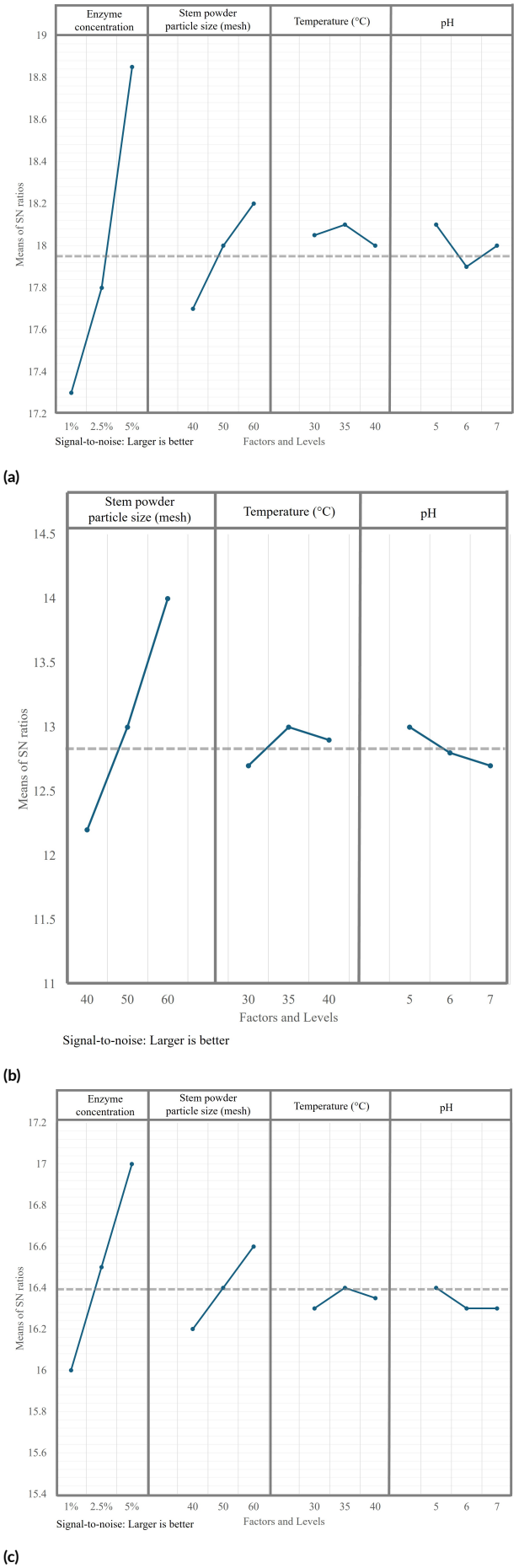
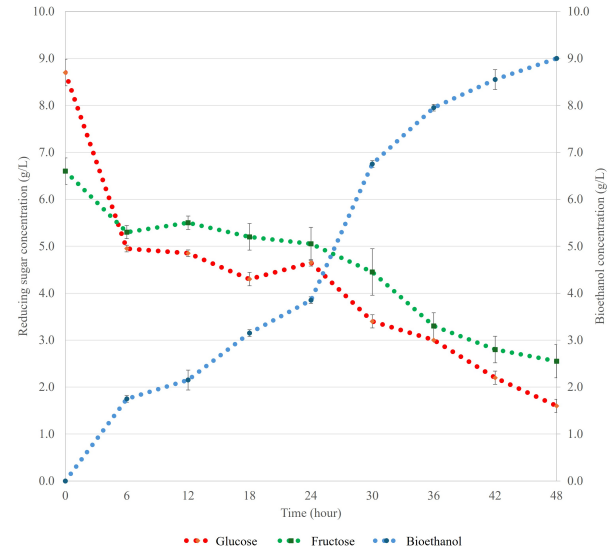
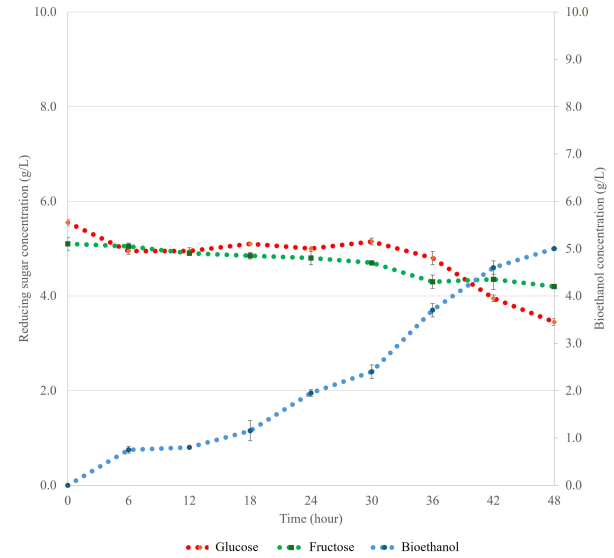


FIGURE 1 Correlation between SNR values and optimization factors in bioethanol production (a) using enzymes and steam treatment; (b) without enzyme; (c) without steam treatment.



(a)



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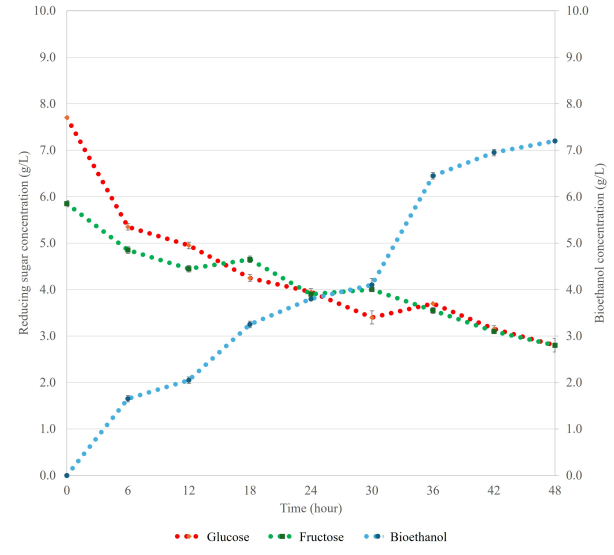


(c)

FIGURE 2 Correlation between reducing sugar consumption and bioethanol production during Simultaneous Saccharification and Fermentation (a) using enzymes and steam treatment; (b) without enzyme; (c) without steam treatment.

3.2. Discussion

3.2.1 Effect of optimization factors on bioethanol production in SSF process

In the simultaneous saccharification and fermentation (SSF) process, enzyme concentration and banana powder particle size have been identified as critical factors significantly impacting bioethanol production. Extensive experimental data reveal that the optimal settings for these factors are an enzyme concentration of 5% (v/v), a sieve pore size of 60 mesh, a temperature of 35 °C, and a pH of 5.00. Under these conditions, the enzyme concentration of 5% (5 UI/mL) achieved the highest bioethanol yield of 9.0 g/L, while a lower concentration of 1.99% (1.99 UI/mL) resulted in a yield of 6.4 g/L.

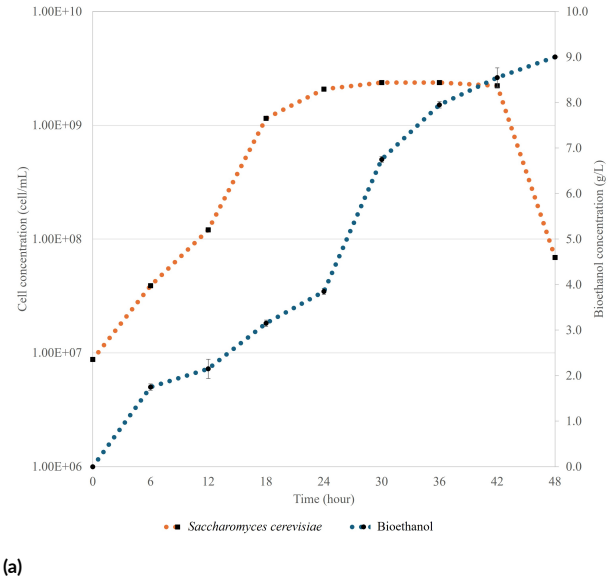
Further findings indicate that 60 mesh banana stem powder facilitated an optimal cellulose concentration of 16%, enhancing the substrate's accessibility to the enzymes. The temperature of 35 °C was confirmed as the most effective for enzymatic activity within yeast cells, while the optimal pH range for maximizing ethanol production through the action of alcohol dehydrogenase was established between 4.00 and 5.00 (Ingale et al. 2014; Suryaningsih and Pasaribu 2015; Uchôa et al. 2021; Carlos Lozano Medina et al. 2024). This enzyme plays a crucial role in converting glucose into ethanol, thus underscoring the importance of maintaining specific process conditions to optimize bioethanol production.

3.2.2 Effect of reducing sugar consumption (glucose & fructose) on bioethanol production in SSF process

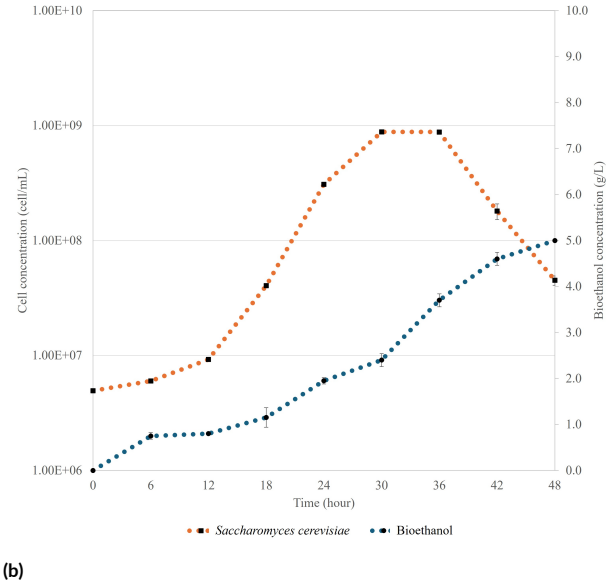
The consumption of glucose and fructose, key substrates in bioethanol production, significantly impacts the output of the simultaneous saccharification and fermentation (SSF) process. Data indicates that there is a notable increase in the consumption of these reducing sugars over a 48-hour fermentation period, which correlates directly with an increase in bioethanol production (refer to Figure 2).

The elevated consumption of reducing sugars can primarily be attributed to the effective enzymatic hydrolysis at an enzyme concentration of 5% (v/v), combined with the pretreatment of banana stem powder via steam treatment and a reduction in the sieve pore size to 60 mesh (Figure 2a). While the absence of steam treatment still resulted in increased sugar consumption (Figure 2c), the effect was less pronounced compared to scenarios where both enzymes and steam treatment were employed. Notably, setups lacking enzyme use (Figure 2b) displayed only a marginal increase in reducing sugar levels, underscoring the enzymes' critical role in breaking down complex carbohydrates into fermentable sugars.

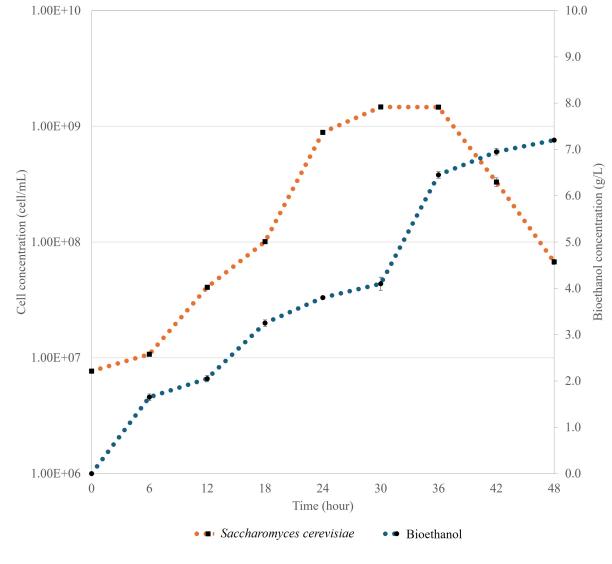
During the initial 24 hours of fermentation, there was a consistent decrease in glucose levels alongside an increase in ethanol production, supporting the understanding that *Saccharomyces cerevisiae* efficiently metabolizes glucose into ethanol (Faizal et al. 2021). The fluctuations in



(a)

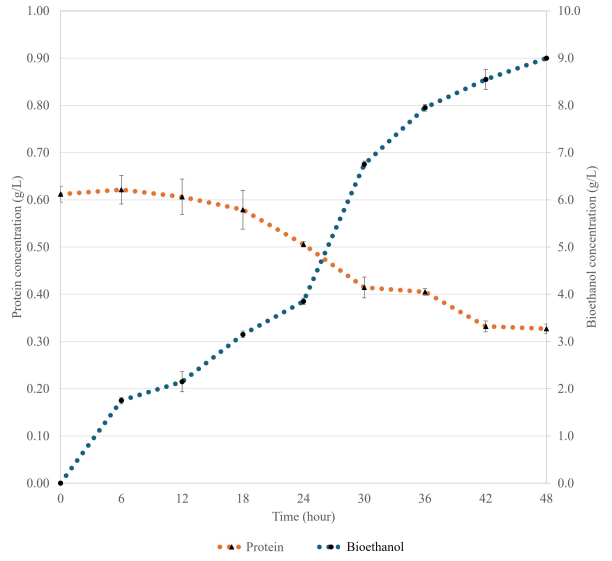


(b)

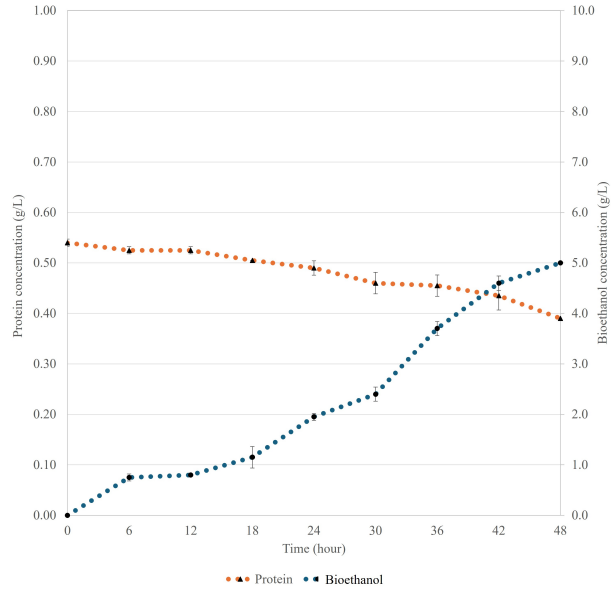


(c)

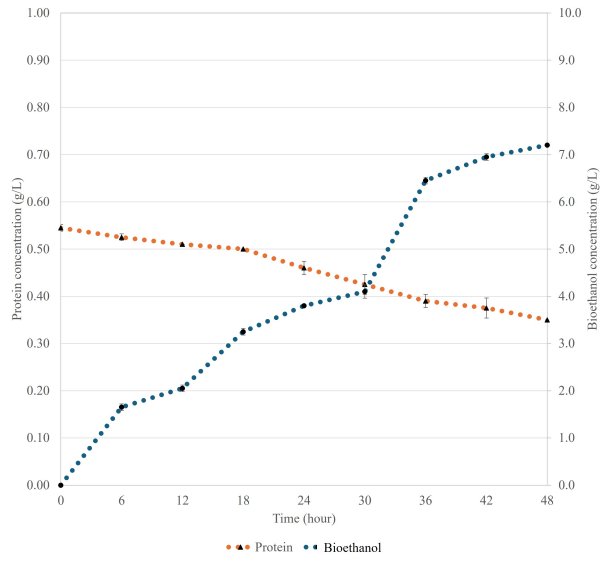
FIGURE 3 Correlation between *Saccharomyces cerevisiae* growth kinetics and bioethanol production during SSF process (a) using enzymes and steam treatment; (b) without enzyme; (c) without steam treatment.



(a)



(b)



(c)

FIGURE 4 Protein concentration with bioethanol production during SSF process (a) using enzymes and steam treatment; (b) without enzyme; (c) without steam treatment.

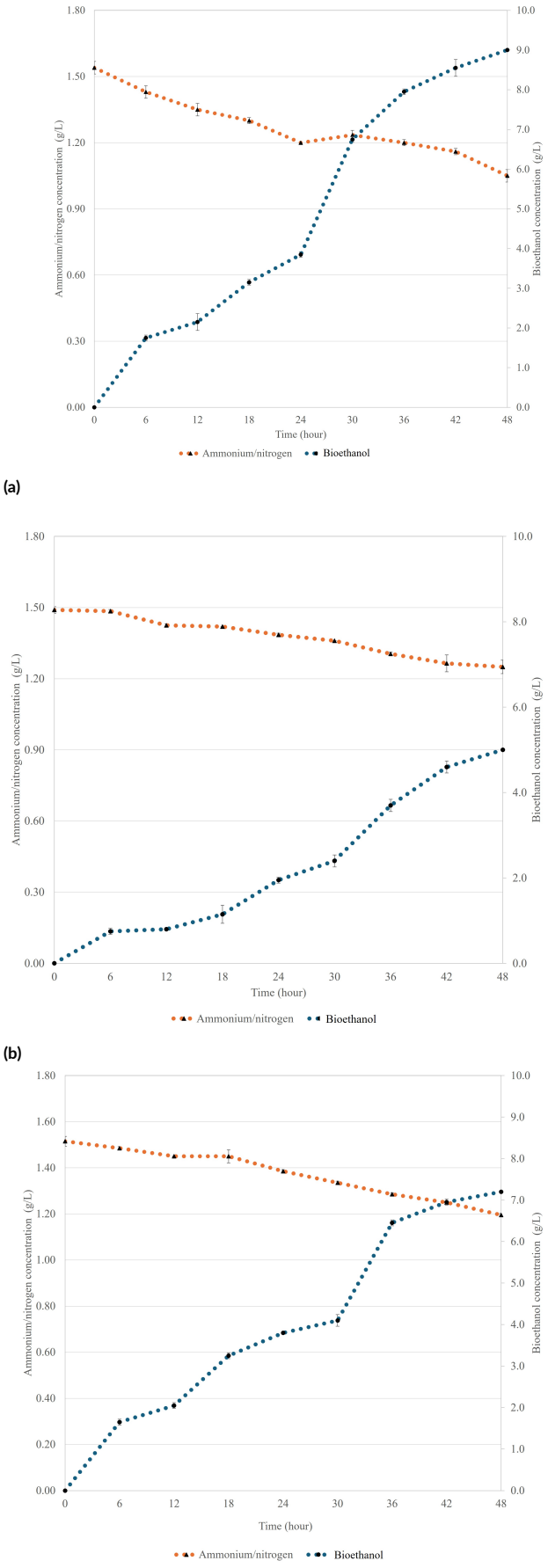


FIGURE 5 Ammonium concentration with bioethanol production during SSF process (a) using enzymes and steam treatment; (b) without enzyme; (c) without steam treatment.

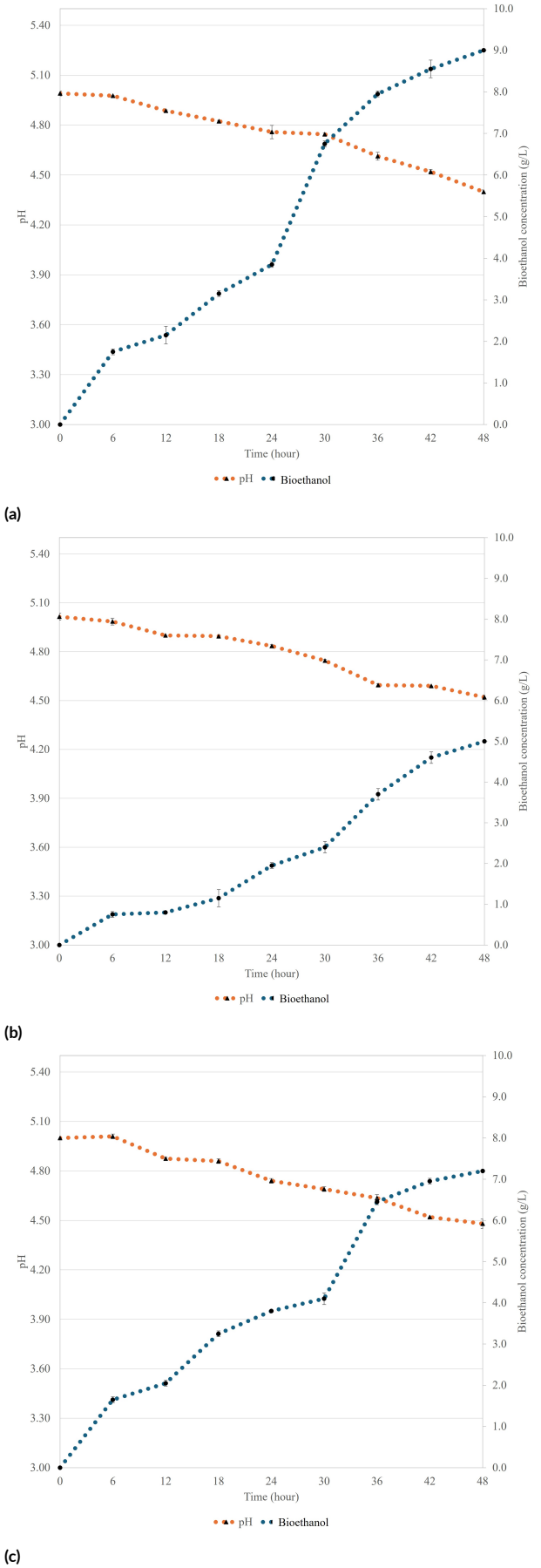


FIGURE 6 pH profile of bioethanol production during SSF process (a) using enzymes and steam treatment; (b) without enzyme; (c) without steam treatment.

sugar consumption across the fermentation timeline may be linked to the dynamic interaction between the cellulase and xylanase enzymes' conversion rates and the yeast's growth kinetics. These interactions potentially explain the variability in reducing sugar availability and subsequent bioethanol yields during the process.

3.2.3 Correlation between *Saccharomyces cerevisiae* growth kinetics and bioethanol production

During the simultaneous saccharification and fermentation (SSF) process, the growth kinetics of *Saccharomyces cerevisiae* shows a direct proportional relationship to the increase in bioethanol production. The application of enzymes and steam treatment significantly influences this growth, as evidenced in the experiment where yeast cell biomass began to increase at the 6th hour under the conditions of 5% (v/v) enzyme concentration, 60 mesh sieve pore size, 35 °C temperature, and pH 5.00 (Figure 3a). In this setup, *Saccharomyces cerevisiae* entered the exponential or logarithmic growth phase from 0 to 18 hours and transitioned to the stationary phase from 18 to 42 hours. The specific growth rate (μ) was calculated at 0.19 per hour, with a generation time of 3.71 hours. The yield of the product formed per cell biomass was 2.85×10^{-9} g/cell per mL, and the product formation rate was 0.23 g/hour.

In contrast, the absence of enzymes (Figure 3b) led to a delayed increase in yeast cell biomass, which only began at 18 hours under the same physical conditions. The yeast experienced a prolonged lag phase from 0 to 12 hours, attributed to slower substrate conversion required for growth, indicating the critical role enzymes play in facilitating faster substrate availability. The growth rate in this condition was 0.17 per hour, with a generation time of 4.01 hours, and the product yield was slightly lower at 2.74×10^{-9} g/cell per mL with a formation rate of 0.08 g/hour.

Furthermore, the absence of steam treatment (Figure 3c) resulted in a delay in the onset of significant biomass increase to the 12th hour, underlining the importance of steam treatment in making cellulose substrates readily available for conversion to glucose. This setup without steam showed a static lag phase during the initial 0 to 6 hours. The growth rate without steam treatment was 0.18 per hour with a generation time of 4.00 hours, and the yield was 2.80×10^{-9} g/cell per mL with a production rate of 0.14 g/hour.

3.2.4 Impact of protein concentration on bioethanol production in SSF process

Protein plays a pivotal role in the simultaneous saccharification and fermentation (SSF) process, serving as a crucial nutrient that supports the metabolic activities of *Saccharomyces cerevisiae*. This yeast utilizes proteins primarily as a source of nitrogen, which is essential for its growth and fermentation activity. The presence of adequate protein in the fermentation medium, derived from the banana stem substrate, ensures that Saccharomyces cerevisiae has access to the necessary nutrients to thrive and produce ethanol efficiently (see Figure 4).

Observations during the SSF process show that within the first 24 hours of fermentation, there is a noticeable decrease in protein content, coinciding with an increase in ethanol production. This inverse relationship highlights the yeast's consumption of protein as it converts available sugars into ethanol. After the initial 24-hour period, the levels of both protein and ethanol stabilize, indicating that the primary metabolic use of protein occurs early in the fermentation process. This stabilization suggests that once the yeast has utilized the available nitrogen source to support its initial growth and start the fermentation, the demand for protein does not increase further.

The consistent protein content beyond the first 24 hours supports the conclusion that Saccharomyces cerevisiae effectively utilizes the protein content from banana stems, confirming the substrate's suitability as a nitrogen source for bioethanol production. This dynamic underscores the importance of protein in enhancing the efficiency and yield of bioethanol during the SSF process, as nitrogen availability is directly linked to the metabolic capacity and health of the yeast cells.

3.2.5 Influence of ammonium concentration on bioethanol production in SSF process

Ammonium plays a critical role in the simultaneous saccharification and fermentation (SSF) process, serving as a vital nitrogen source that supports the growth and metabolic activities of *Saccharomyces cerevisiae*. As an essential component, ammonium directly contributes to the nutritional environment required by the yeast, facilitating its ability to ferment glucose into ethanol effectively.

Experimental observations indicate that during the SSF process, the concentration of ammonium in the medium decreases over a period of 48 hours, coinciding with an increase in bioethanol production (see Figure 5). This trend suggests that *Saccharomyces cerevisiae* utilizes ammonium primarily during the early stages of fermentation, absorbing it to support cellular growth and metabolism, which in turn enhances ethanol production. The reduction in ammonium levels is a positive indicator of yeast activity, as it reflects the conversion of available nitrogen into biomass and metabolic byproducts, including ethanol.

This dynamic between ammonium consumption and ethanol production underscores the importance of optimizing ammonium levels within the fermentation medium to maximize bioethanol yield. Ensuring sufficient ammonium at the onset of the SSF process is crucial for supporting robust yeast performance and achieving higher ethanol outputs (Faizal et al. 2021).

3.2.6 pH dynamics during bioethanol production in SSF process

The pH profile is a crucial factor that significantly influences the growth kinetics of *Saccharomyces cerevisiae* and consequently affects bioethanol production during the simultaneous saccharification and fermentation (SSF) process. Optimal pH levels are essential for maintaining the metabolic activity and overall health of the yeast, ensuring efficient fermentation.

Research findings indicate that the optimal pH range for *Saccharomyces cerevisiae* during the SSF process is between 4.41 and 5.00 (see Figure 6). Within this pH range, the yeast exhibits enhanced growth and metabolic activity, which directly correlates with higher bioethanol yields. Maintaining this pH range ensures that the yeast operates under favorable conditions, promoting the efficient conversion of sugars into ethanol.

The dependency of bioethanol production on pH levels highlights the importance of closely monitoring and adjusting the pH during the SSF process. Deviations from the optimal pH range can lead to suboptimal yeast performance, reduced fermentation efficiency, and lower ethanol yields. Therefore, controlling the pH within the specified range is crucial for maximizing bioethanol production and achieving consistent, high-quality results in the SSF process.

4. Conclusions

Steam treatment is a critical process in bioethanol production, as it effectively breaks down lignin and hemicellulose compounds, enhancing the availability of cellulose for enzymatic hydrolysis. This pretreatment step significantly influences the efficiency and yield of bioethanol production during the simultaneous saccharification and fermentation (SSF) process.

Key factors that affect the quality and quantity of bioethanol production include enzyme concentration and sieve pore size. Optimal conditions for maximizing bioethanol yield were determined through a series of experiments. The highest bioethanol production was achieved with an enzyme concentration of 5% (v/v), 60 mesh banana stem powder, a temperature of 35 °C, and a pH of 5.00. Under these conditions, the maximum bioethanol yield reached 9.0 g/L.

Furthermore, the product yield per cell biomass was found to be 2.85×10^{-9} g/cell per mL, with a product formation rate of 0.23 g/hour. These findings underscore the importance of optimizing enzyme concentration and sieve pore size, alongside maintaining optimal temperature and pH levels, to achieve high-efficiency bioethanol production. The combination of steam treatment and these optimized factors results in a significant improvement in the overall bioethanol yield, demonstrating the effectiveness of the integrated SSF process.

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Authors' contributions

AS, NP, and DIA designed the study. AS, NP carried out the laboratory work. AS, NP, DIA analyzed the data. AS, NP wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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