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Research Article

A Single-Reactor System for Simultaneous Pretreatment and Fermentation of POME for Bioethanol Production

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The objective of this study was to develop an efficient method for enhancing the production of bioethanol in a single-reactor system (SRS) by implementing acid–base pretreatment and enzymatic hydrolysis techniques, thereby eliminating differentiation and removal processes. The aim was to establish a process for bioethanol synthesis using hydrolyzed palm oil mill effluent (POME) and a locally sourced cellulase enzyme. The pretreatment and enzymatic hydrolysis methods were successfully optimized, resulting in a maximum yield of reducing sugars of 26.6 g/L (53.14%). To achieve the highest bioethanol yield, fermentation was carried out using both the one-factor-at-a-time (OFAT) and face-centered central composite design (FCCCD) approaches. The OFAT approach was employed to obtain the maximum bioethanol production, which yielded 6.75% *v*/*v* of bioethanol from the hydrolyzed POME using the same bioreactor. In the case of the FCCCD process, the optimal conditions led to a bioethanol production of 7.64% *v*/*v* during the fermentation stage. Kinetic analyses of the bioethanol produced revealed a specific growth rate (μ) of 0.198 h^{-1} and a specific product formation rate (q_p) of 0.239 h^{-1} after 3 days of fermentation. These findings highlight a promising strategy for efficient management of POME through the production of biofuels, which could contribute to the economic growth of the country.

Keywords: bioconversion process; bioethanol production; OFAT; POME; reducing sugar; SRS

1. Introduction

The increasing demand for palm oil is expected to result in a significant amount of wastewater generated by the palm oil industry. This wastewater, known as palm oil mill effluent (POME), is primarily produced during the processes of oil extraction, cleaning, and washing [1]. POME has adverse effects on surface waters due to its high levels of chemical oxygen demand (COD) (ranging from 15,000 to 100,000 mg/L), acidic pH (3.5–4), substantial biomass content (60%), oil and grease (4000 mg/L), total nitrogen (180–1400 mg/L), biochemical oxygen demand (BOD) (10,250–43,750 mg/L), total solids (11,500–79,000 mg/L), water content (95%–96%), and total suspended solids (5000–54,000 mg/L) [2, 3].

To address the treatment of POME, the majority of palm oil refineries rely on anaerobic digestion methods [4]. Locally and internationally, more than 85% of palm oil companies utilize ponding techniques for effluent treatment, while a smaller portion opt for open digesting tanks [5]. These conventional treatment approaches often require complex stabilization periods and advanced treatment facilities. In laboratory settings, high-rate anaerobic bioreactors such as the upflow anaerobic sludge–fixed film (UASFF) reactor [6, 7], upflow anaerobic sludge blanket (UASB) reactor [8], and upflow anaerobic filtration [9] have been

employed. Influent treatment has also been explored using continuous stirred tank reactors (CSTRs) and anaerobic contact digesters [10]. POME was previously studied treated using an evaporation approach [11], an aerobic-activated sludge reactor [10], and membrane filtering technology [12] in addition to anaerobic digestion. According to a recent study, the factory anaerobic and pond treatment systems use unidentified microorganisms which were essential for active ingredient and contaminant anaerobic degradation. Unexpected anaerobic decomposition standard operating strategies also induce digestive issues, resulting in remediation collapse. Furthermore, POME has an effect on microbial sustainability; Mohamad-Zainal et al. [10] reported a conversion in low nucleic acid (LNA) to high nucleic acid (HNA) bacterium in a riverside as a consequence of POME release into the atmosphere.

Significantly, POME pretreatment was a commercialized alternate solution source of environmentally friendly technology that sometimes captures over 40% of CO_2, almost 70% of CH_4, 10% of H_2, and 0.1% of H_2S, and its utilization as biofuels was increasing favor internationally [11]. Pretreatment, enzymatic hydrolysis, and distillation were also unit processes that contribute significantly to the cost of producing bioethanol. Present efforts for research and development were also directed at upgrading these system components in order to make them more cost-effective. Treatment methods had a high capability to be utilized in biofuels, but their complicated structure throughout enzymatic hydrolysis from cellulose and hemicellulose [3] makes them difficult to employ. To increase the output of reducing sugars during hydrolysis, treatment was indeed necessary. The existence of increasingly nonbiodegradable lignin in the lignocellulose material and the reduced digestibility of crystalline structure cellulose and hemicellulose were major barriers to the utilization of POME without pretreatment during the enzymatic scarification process. The preparation lowers cellulose crystallinity, yields morphological cellulose, eliminates or degrades lignin, and necessitates either complete or partial hemicellulose hydrolysis [12]. Overall pretreatment also must yield substrates that were readily digested and had a higher specific surface area. Conversely, hydrolytic enzymes derived from bacteria and fungi efficiently use cellulose [13]. Pretreatment overall performance was especially essential since it impacts the response of enzyme reagents. Appropriate and efficient pretreatment reduces metabolic activity reduction and consequently enhances the rate of material breakdown. Current studies concentrate on increasing enzymatic reactions by investigating novel microbes with cellulolytic and hemicellulolytic function or catalytic doses that transform lignocellulose cellulose to biofuel [14]. Enzymes were also reused, which decreases the budget of the hydrolysis process. The usable contact area of cellulosic biomass to catalysts affects hydrolysis reaction and catalyst concentration. This improves cellulose breakdown performance. This was due to the surfactant being deposited on the lignin layer rather than the catalyst and therefore not deactivating the enzyme [15].

The process efficiency, growth rate, and inhibitory impact of ethanol on complex bacteria utilized for the conversion of hexoses and pentoses to biofuel and CO_2 were all studied. The aim of digestion was to synthesize hexose and pentose carbohydrates to biofuel as effectively as possible by fermented microbes also including *Saccharomyces* [16]. Based on preliminary experiments, the optimization of the fermentation process focuses on key variables such as pH, inoculum percentage, and fermented sugar content. These variables were chosen due to their significant impact on the efficiency and yield of the fermentation process. Inoculum percentage affects the microbial population and activity, pH influences the enzymatic and microbial environment, and fermented sugar content serves as the primary carbon source for microbial metabolism. By optimizing these variables, the study is aimed at enhancing the overall performance of the fermentation process [17]. Microbes that metabolize xylose effectively in controlled circumstances exhibit poor soluble protein yields from lignocellulosic materials. Biodegradation causes stress conditions including biofuel buildup, progressive pH decline, anaerobic condition growth progression, and nutritional restriction. Recirculating vapor/liquid diffusion coefficient was frequently utilized in traditional extraction processes. From contaminated biomass-based streams, formulation may extract concentrated volatile compounds including biofuel [18]. These streams were contaminated by residual organic polysaccharides (cellulose and hemicellulose), ash, and chemicals derived from lignin degradation. Fermented biofuel was polluted and arrives in minute parts. During liquid–liquid extraction, biofuel was differentiated from the mixture's additional ingredients. Reconsideration enables biodiesel yield and detoxification. Therefore, extremely high-concentration (99.7% by volume) biofuel can only be obtained following dehydration and drying [19].

This study is aimed at advancing bioethanol production from POME by introducing a novel single-reactor system (SRS) that integrates simultaneous pretreatment and fermentation processes. Unlike traditional methods that involve separate stages for pretreatment and fermentation, this approach is aimed at streamlining operations and reducing costs associated with POME treatment. By optimizing fermentation parameters and utilizing locally produced enzymes derived from PKC by *Trichoderma reesei*, the study is aimed at achieving higher bioethanol yields efficiently from POME. POME was chosen due to its rich content of carbohydrates, minerals, and nitrogen, making it an ideal substrate for bioconversion. Pretreatment is employed to enhance the breakdown of hemicellulose and lignin, thereby improving cellulose accessibility. These methods are widely used in waste treatment where efficient breakdown is crucial. Through the optimization of inoculum and sugar concentrations, this research is aimed at enhancing the efficiency of the fermentation process. Inoculum concentration is a critical factor as it influences the microbial population and activity, thereby affecting the overall fermentation rate and yield. Similarly, sugar concentration is pivotal as it serves as the primary carbon source for microbial metabolism. Additionally, substrate loading is considered an essential independent variable in this study. Substrate loading determines the amount of material available for fermentation, which can

impact the efficiency and scalability of the process. By optimizing these variables, the study seeks to identify the conditions that maximize yield and productivity. This innovative single-step fermentation process not only addresses environmental challenges posed by POME but also offers practical and economic benefits in biotechnology industries. This work builds on preliminary studies that have explored POME as a carbon source for bioethanol production using *Saccharomyces cerevisiae*, investigating and expanding upon the acid–base–enzyme mechanism. Additionally, kinetic studies were conducted to assess the bioethanol production process, underscoring its feasibility and effectiveness in sustainable biofuel generation.

2. Material and Methods

2.1. Materials. POME was taken at the discharge point to the anaerobic ponding process from Sime Darby Plantation Sdn. Bhd., Carey Island, Malaysia. The material was kept in a cool chamber (4°C) to avoid fungal development. The phenol sulfuric acid technique [20] was utilized to determine the reducing sugar and total sugar using a spectrophotometer at 490 nm (Shanghai Spectrum Instruments, China), and pH was monitored using pH meters (Hach, United States). Analytical grade chemicals used include sodium sulfite (Merck, Germany); sodium potassium tartrate (Merck, Germany); hydrochloric acid (Merck, Germany); carboxymethyl cellulose and 3,5-dinitrosalicylic acid (Merck, Germany); acetone (Hmb GLOBAL Chemical, Germany); bacteriological agar, bacteriological peptone, yeast extract peptone dextrose (YPD), and potato dextrose agar (PDA) (OXOID Ltd, England); citric acid monohydrate and phenol (R&M Chemical, United Kingdom); COD HR reagent (300–1500 mg/mL) (Hach, United States); ethanol 95% and glucose (anhydrous) (Hmb GLOBAL Chemical, Germany); potassium dichromate (The Science Company, United States); sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH) (Fisher Scientific, United Kingdom); Tween 80 (Fisher Scientific, United Kingdom); YPD (Sigma-Aldrich, Germany); and *T. reesei* (Novozyme, Denmark). Data are the average of three replicates.

2.2. Enzyme Preparation. The cellulase enzyme was locally produced in the laboratory at International Islamic University Malaysia (IIUM). For production, PKC (Sime Darby Plantation Sdn. Bhd., Carey Island, Malaysia) was used as a basal medium for *T. reesei* with a medium composition of 0.2% T80 as described by Deb et al. [21].

2.3. Yeast Culture. S. cerevisiae strains were obtained from the laboratory stock and transferred to PDA plates for subculturing. A viable colony was selected from an agar plate and transferred to a 250-mL Erlenmeyer flask containing 100 mL of YPD medium, which consisted of 10 g/L yeast extract and 20 g/L peptone. The flask was then placed in an incubator set at 37°C. The cells were allowed to grow at a concentration of 10^8 cells/mL, with incubation carried out at 30°C for a period of 24–48 h, while agitating the flask at 150 rpm.

2.4. Preparation of Inoculum. Alam et al. [20] utilized a technique to prepare a starter culture for enzyme synthesis. In order to maintain consistent mycelium concentrations, each 7-day-old *T. reesei* fungal culture plate was gently rinsed with approximately 25 mL of sterile boiled water using a bent glass rod. The fungal samples, containing microcolonies, were then separated from the bacterial solution by filtering them through Whatman No. 1 chromatography paper. After determining the concentration of spores (ranging from 50×10^8 to 500×10^8 spores/mL) using a hemocytometer, the solution was filtered and transferred to a 250-mL Erlenmeyer flask for use as an inoculum (S1).

For the preparation of the inoculum for bioethanol production, *S. cerevisiae* yeast culture plates were used. Each plate was rinsed with approximately 10 mL of sterile boiled water using a bent glass rod. Subsequently, 10 mL of colony mycelium was added to 90 mL of YPD growth medium containing 20 g of peptone and 10 g of yeast extract in a 250-mL Erlenmeyer flask. The inoculated sample was then cultured for 2 days at room temperature (30 ± 2°C) with agitation at 150 rpm to facilitate bacterial development. The cell concentration in the inoculum was determined to be 10^8 cells/mL for future utilization in the bioconversion process.

2.5. BioE Determination by Chromic Acid Method. The total ethanol concentration in the medium was quantified using the chromic acid technique, based on the method outlined by Deb et al. [21]. The calorimetric determination of ethanol involved the following steps: 1 mL of various ethanol concentrations was combined with 4 mL of distilled water in separate test tubes. To each tube, 5 mL of chromic acid was added and thoroughly mixed. The test tubes were then placed in a water bath at 60°C for 20 min and subsequently allowed to cool. The absorbance of the solutions was measured at 584 nm using a spectrophotometer. By constructing a standard curve using absolute ethanol, the ethanol concentration in the samples was determined.

2.6. Determination of Bioethanol by GC/MS. The objective of the study was to analyze the fermentation of a hydrolysate POME–based medium by *S. cerevisiae* to obtain biofuel samples for GC/MS extraction. Initially, the fresh biofuel solution underwent centrifugation to separate distinct solubilized colloidal solids and materials from the solution. The resulting filtrate was used as the input reagent for GC-MS 3700 (Hangzhou EXPEC Technology, China) analysis. Acetone and discrimination chromatography were employed to purify the cellulosic ethanol solution. The concentration of bioethanol present was determined using chromatographic techniques.

The experimental results indicated the successful separation of bioethanol using the DBWAX phase, despite the bioethanol being connected to the GC/MS polymer. The composition of bioethanol varied, and the extracted amount was determined to be 11.6585% *v*/*v* based on the experimental data (Face-Centered Central Composite Design (FCCCD) Sample Run 4). These concentrations of bioethanol have been widely utilized in various studies [21].

2.7. Preparation of Hydrolyzate by Simultaneous Pretreatment and Hydrolysis of POME

2.7.1. Acid–Base–Enzyme Pretreatment Process. The raw POME pretreatment involved a two-step process with sulfuric acid (1% *v*/*v*, 4% dose) followed by sodium hydroxide treatment. Acid-treated samples were incubated at room temperature for 60 min. Optimal conditions were determined by monitoring reducing sugar production as discussed in the previous paper by Deb et al. [21]. Subsequently, sodium hydroxide pretreatment (3% *w*/*v*) at pH 6 enhanced reducing sugar production. Fermentation occurred for 1 h at 30 ± 2°C and 150 rpm as discussed in the previous paper by Deb et al. [21].

Inhibitors can form during pretreatment processes, particularly in methods involving acids or alkalis. These inhibitors are often byproducts of the breakdown of lignocellulosic biomass components such as hemicellulose and lignin. Common inhibitors include furfurals (like furfural and 5-hydroxymethylfurfural) and organic acids (such as acetic acid and formic acid), which can inhibit subsequent enzymatic hydrolysis and fermentation processes. Managing inhibitor formation is crucial for optimizing bioethanol production from biomass sources like POME, as it can affect overall ethanol yields and process efficiency. Various strategies, such as detoxification steps or enzyme engineering, are employed to mitigate the impact of inhibitors in biofuel production processes.

2.7.2. Enzymatic Hydrolysis Process. The enzymatic degradation process was conducted using the same 250-mL Erlenmeyer flask that was previously used for pretreatment. A cellulase enzyme with an activity of 40 CMC μmol-min^{-1} mL^{-1} was added to the flask containing 80 μmol-min^{-1} mL^{-1} of pretreated POME to assess the rate of enzymatic hydrolysis. Several parameters were investigated to optimize the process conditions, including the cellulase enzyme dose (80 μmol min^{-1}) for a duration of 60 min, enzyme pH (5), and hydrolysis time (18 h). The flasks were maintained at an ambient temperature of 30 ± 2°C and shaken at 150 rpm. Samples were collected at 18-h intervals, subjected to vortexing at 5000 rpm and then cooled at 4°C for 20 min. The resulting homogenate was tested for the presence of reducing sugars as discussed in the previous paper by Deb et al. [21]. In addition to optimizing the substrate concentration, the cellulase enzyme dose was also optimized using the FCCCD approach.

2.7.3. Optimization Strategy. In this experiment, the experimental studies were conducted using two different optimization methods. The first method utilized was the OFAT (one-factor-at-a-time) technique, which was aimed at determining the potential optimum parameters for the fermentation process. The parameters studied included fermentation pH, fermentation time, and inoculum percentage (*v*/*v*). The OFAT analysis technique was used to investigate the influence of these parameters on the bioethanol production. Table 1 outlines the OFAT experimental design used to optimize various parameters in a fermentation process using hydrolysate POME as the substrate. Each factor was varied within a specific range while keeping other conditions constant. For example, the fermentation pH was tested from 4 to 8 while maintaining 100 mL of hydrolysate POME, an inoculum dose of 3%, a temperature of 30°C, agitation at 150 rpm, substrate loading at 5%, and a fermentation time of 7 days [21]. Similarly, other factors like inoculum concentration, temperature, agitation speed, substrate loading, and fermentation time were varied within their respective ranges, with the remaining parameters held constant.

The specific ranges tested for each factor were designed to identify the optimal conditions for the fermentation process. Inoculum concentration ranged from 1% to 6% *v*/*v*, with other parameters fixed at a pH of 7, temperature of 30°C, agitation at 150 rpm, and fermentation time of 7 days [21]. Temperature was varied from 25°C to 45°C under similar constant conditions. Agitation speed ranged from 50 to 400 rpm, substrate loading from 0.5% to 6%, and fermentation time from 0 to 9 days. These experiments were aimed at determining the best combination of conditions to maximize the efficiency and yield of the fermentation process [3, 21].

To optimize the independent variables identified from preliminary experiments—pH, inoculum percentage, and fermented sugar content—a FCCCD was employed. This design allows for a comprehensive exploration of the interactions between these variables and their impact on the fermentation process. The selection of these variables is grounded in prior experimental findings, ensuring a targeted and effective optimization strategy. To further characterize the relationship between the independent variables (fermentation pH, fermentation time, and inoculum percentage) and the dependent variable (bioethanol production), the FCCCD was employed. This design allowed for the exploration of the response surface and the identification of suitable conditions for maximizing bioethanol production. The FCCCD analysis helped in understanding the interaction between the variables and provided valuable insights for process optimization.

The data obtained from the experiments were calculated as the means of three replicates. To determine the statistical significance, analysis of variance (ANOVA) was performed, followed by the least significant difference test using statistical software. The design of experiments (DOE) for this study was conducted using Design-Expert Version 10, which facilitated the systematic and efficient exploration of various experimental conditions and their effects on bioethanol production. To optimize the independent variables, namely, pH, inoculum percentage, and fermented sugar content, the FCCCD was employed. The main objective of this design was to study the effects of these variables on the production of bioethanol. In order to model the response surface, six center points were included in the design. The response surface methodology (RSM) was utilized, which combines statistical and mathematical tools to design, improve, and optimize processes. RSM is a powerful technique that is used when multiple variables, known as independent variables, influence a set of parameters or production values, referred to as the response of interest. The primary goal is to

Table 1: OFAT factors designed for fermentation.

Factor	Range tested	Fixed parameters
Fermentation pH	4–8	100 mL hydrolysate POME, inoculum dose 3%, temperature 30°C, agitation 150 rpm, substrate loading 5%, and fermentation time 7 days
Inoculum (% *v*/*v*)	1–6	100 mL hydrolysate POME, pH 7, temperature 30°C, agitation 150 rpm, substrate loading 5%, and fermentation time 7 days
Temperature (°C)	25–45	100 mL hydrolysate POME, pH 7, inoculum dose 3%, agitation 150 rpm, substrate loading 5%, and fermentation time 7 days
Agitation (rpm)	50–400	100 mL hydrolysate POME, pH 7, inoculum dose 3%, temperature 30°C, substrate loading 5%, and fermentation time 7 days
Fermentation time (days)	0–9	100 mL hydrolysate POME, pH 7, inoculum dose 3%, temperature 30°C, agitation 150 rpm, and substrate loading 5%

optimize this response by manipulating the variables. It provides a systematic approach for modeling and analyzing complex problems.

In the FCCCD, the response surface was created by conducting experiments with three levels for each variable: high, medium, and low. A total of 20 experimental runs were performed to study the relationship between the variables and the response of interest, which in this case was the production of bioethanol. This design allowed for a comprehensive exploration of the experimental space and facilitated the identification of optimal conditions for maximizing bioethanol production.

2.7.4. A Single-Reactor Bioethanol Production. Figure 1 illustrates a bioethanol production process conducted in a SRS. The hydrolyzed POME was converted into ethanol within the same 250-mL Erlenmeyer flask used for pretreatment and hydrolysis. To ensure sterility and prevent contamination, the samples were subjected to proper sterilization at 121°C for 15 min. The flasks were then cooled in a laminar airflow cabinet.

During the fermentation process, several parameters were optimized to enhance the production of bioethanol. The fermentation pH was varied within the range of 4 to 8, the inoculum percentage was adjusted between 1% and 6%, and the fermentation time spanned from 1 to 7 days. The flasks were placed in a rotary shaker operating at 150 rpm and maintained at a temperature of 30°C, as previously determined by Alam, Kabbashi, and Hussin [22].

After fermentation, the fermented samples were collected in 50-mL centrifuged tubes and subjected to straining for 15 min at 5000 rpm and a temperature of 4°C. The resulting homogenate was analyzed to determine the bioethanol content.

2.8. Analysis of the Fermentation Kinetic Parameters. In order to evaluate the impact of fermentation duration on bioethanol production and the kinetics of bioethanol production and biomass development, an experiment was conducted. Optimal medium and saccharification process parameters were used in this study. Several factors were assessed during the experiment, including bioethanol production, biomass growth measured in terms of TSS, and the content of reducing sugars. These factors were monitored at regular intervals of 6 h up to a duration of 72 h.

The yield of bioethanol production (Y_p/s) is typically calculated as the ratio of bioethanol produced (Y_p) to the total reducing sugars consumed (s). Equation (1) is

$$Y_p/s = \frac{\text{bioethanol produced } (Y_p)}{\text{total reducing sugars consumed } (s)} \quad (1)$$

The yield of product formation from biomass (Y_p/x) represents the efficiency of converting biomass into bioethanol. It is calculated as Equation (2):

$$Y_p/x = \frac{\text{bioethanol produced } (Y_p)}{\text{biomass produced } (x)} \quad (2)$$

The yield of biomass formation from reducing sugar consumption (Y_x/s) indicates how efficiently biomass is formed from the consumption of reducing sugars. Equation (3) is

$$Y_x/s = \frac{\text{biomass produced } (x)}{\text{total reducing sugars consumed (s)}} \quad (3)$$

The specific rate of product formation (q_p) describes how quickly bioethanol is produced per unit biomass or per unit time. It can be calculated as Equation (4):

$$q_p = \frac{d\,[\text{bioethanol}]}{dt} \times \frac{1}{[\text{biomass}]} \quad (4)$$

The specific growth rate (μ) represents the rate at which biomass concentration increases over time. It is often calculated using Equation (5).

$$\mu = \frac{dX}{dt} \times \frac{1}{X} \quad (5)$$

where X is the biomass concentration and dX/dt is the rate of change of biomass concentration over time. These equations are fundamental in understanding the dynamics of bioethanol production and biomass growth during fermentation processes,

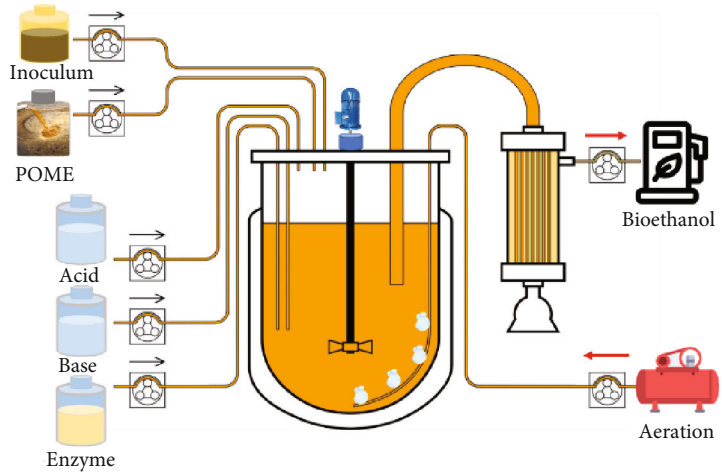

Figure 1: Diagram of a single-reactor bioethanol production process.

providing insights into process efficiency and performance. From the experimental data, various parameters were estimated to analyze the fermentation process. These included the yield of bioethanol production based on the total consumption of reducing sugars (Y_p/s), the yield of product formation from biomass (Y_p/x), and the yield of biomass formation based on reducing sugar consumption (Y_x/s). Additionally, the specific rate of product formation (q_p) and the specific growth rate (μ) were determined. By analyzing these parameters, the researchers were able to gain insights into the kinetics of bioethanol production and biomass development during the fermentation process. These findings provide valuable information about the efficiency and performance of the fermentation process, shedding light on the dynamics of bioethanol production and microbial growth.

2.9. The Error (%). The error (%) represents the percentage difference between the theoretical and experimental bioethanol production, which is typically calculated using Equation (6):

$$\text{Error}\,(\%) = \frac{(\text{experimental bioethanol}\,(\%v/v) - \text{theoretical bioethanol}\,(\%v/v)}{\text{theoretical bioethanol}\,(\%v/v)} \times 100 \quad (6)$$

This formula is applied to each experiment to quantify the deviation between the expected (theoretical) and actual (experimental) bioethanol production percentages, providing insight into the accuracy of the experimental model.

3. Results and Discussion

3.1. Cell Growth and Characterization. Figure 2 depicts the preparation of freshly cultured *S. cerevisiae* for syngas fermentation. In Figure 2 (the strains TNSC1 and TNSC2), it can be observed that the newly cultured microbial cells were grown in slants and petri plates, respectively. In strain TNSC1, the culture can be seen in a petri plate, while in strain TNSC2, it is shown in a slant. These two growth formats are commonly used to cultivate and maintain microbial cells. The SEM images in strain TNSC1 provide a closer look at the *S. cerevisiae* colonies. Strain TNSC2 shows an image of a group colony, where multiple cells have grown together, while strain TNSC1 displays an image of a single colony, focusing on an individual cell.

SEM analysis allows for detailed examination of the morphology and structure of the cells. These images provide valuable information about the appearance and characteristics of the *S. cerevisiae* cells, helping to confirm their identity and suitability for the intended purpose, such as syngas fermentation and bioethanol production as depicted in Figure S1. This specific strain of *S. cerevisiae* was chosen due to its ability to thrive under certain limiting conditions necessary for efficient bioethanol production. Previous studies [23] have utilized this strain and reported high yields of second-generation bioethanol.

To confirm the identity and characteristics of the freshly cultured *S. cerevisiae*, SEM analysis was performed, and the results are shown in Figure 2. The morphological analysis revealed that the cell surface was smooth and the cells had a spherical shape. These SEM images are consistent with the findings reported in the literature by Zhao, Lin, and Chen [24]. Thus, based on this analysis, it can be concluded that the freshly cultured *S. cerevisiae* was suitable for syngas fermentation and well suited for bioethanol production.

Table 2 provides the characteristics of POME in wastewater generated during the processes of palm oil extraction, cleaning, and washing. It is characterized by high levels of COD of 500,000 mg/L, indicating its organic pollutant load. The pH of POME is 4.25, contributing to its acidic nature. POME contains significant biomass content, approximately 60%, along with oil and grease concentrations of about 4000 mg/L. Total nitrogen levels are 196 mg/L, while BOD is 23,750 mg/L, reflecting its high biological oxygen demand. The wastewater also exhibits a substantial total solid content of 59,000 mg/L, with a predominant water content of 95%. Total suspended solids in POME are 54,000 mg/L, highlighting its heterogeneous composition [1–3, 21].

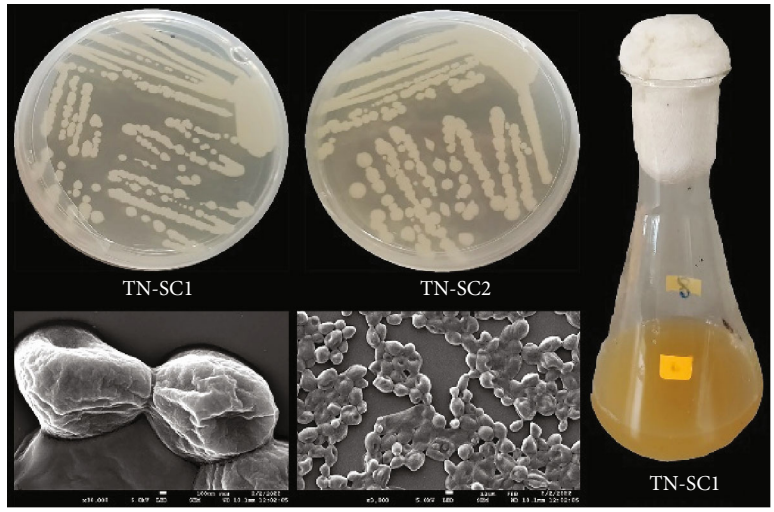

Figure 2: *Saccharomyces cerevisiae* cell growth culture: petri plate. SME image of 10-μm *Saccharomyces cerevisiae* group colony and image of 10-nm *Saccharomyces cerevisiae* single colony.

Table 2: The characteristics of POME.

Characteristic	Value
Chemical oxygen demand (COD)	50,000 mg/L
pH	4.25
Biomass content	60%
Oil and grease	4000 mg/L
Total nitrogen	196 mg/L
Biochemical oxygen demand (BOD)	23,750 mg/L
Total solids	59,000 mg/L
Water content	95%
Total suspended solids	54,000 mg/L
Reducing sugar	2.9 mg/mL

This characterization underscores the complex and variable nature of POME, necessitating effective treatment strategies to mitigate its environmental impact and harness its potential for bioethanol production [21].

POME's composition, including its COD, pH, biomass content, and levels of nitrogen and solids, influences how effectively enzymes can hydrolyze the biomass during pretreatment. Factors such as high COD and biomass content can affect enzymatic digestion efficiency, potentially altering the amount of reducing sugars available for fermentation. Optimizing pretreatment methods tailored to POME's specific characteristics is crucial for maximizing the ethanol production yield.

3.2. Bioethanol Production From POME Hydrolysate Using the OFAT Technique. The investigation was to test the hypothesis that the hydrolyzed POME with *S. cerevisiae* as yeast in a batch bioreactor may have enhanced the bioethanol production. Optimization of the fermentation was done in two steps. In the beginning, three significant parameters were observed in an OFAT design to assess probable optimal levels of the parameters. The parameters were fermentation pH, fermentation day, and inoculum dose with the hydrolysis strategy of the POME developed in the previous sections. Subsequently, the parameters found to be optimum from the OFAT study were further examined by the statistical optimization method, FCCCD.

3.2.1. Effect of Fermentation pH. The pH of the growth medium plays a crucial role in microbial growth and bioethanol production during the LSF (liquid state fermentation) process. Each microorganism has a specific preferred pH range that is optimal for its growth and activity. The OFAT technique revealed a significant positive correlation between bioethanol yield and inoculum pH, indicating that higher pH levels resulted in relatively lower bioethanol synthesis. Based on this observation, a fermentation medium with a pH range of 4 to 9 was chosen for the OFAT investigation, as depicted in Figure 3.

The experimental results demonstrated that adjusting the fermentation medium to pH 6 led to an increased bioethanol production of 7.7% *v*/*v*. Additionally, the findings indicated that bioethanol output gradually increased from pH 4 to pH 5, reaching around 6% *v*/*v*. However, a sharp decline in bioethanol production was observed when the pH of the growth medium exceeded from pH 7 to pH 8. These results highlight the significance of maintaining an optimal pH range in the growth medium to maximize bioethanol production. The findings suggest that a pH of 6 provides favorable conditions for the microorganisms, resulting in higher bioethanol yields. Controlling and adjusting the pH within the appropriate range are essential for optimizing the efficiency of the LSF process and achieving higher bioethanol production. From Figure 3, the dynamic relationship between fermentation time, pH levels, and bioethanol production was observed. As the fermentation period extends, a noticeable increase in bioethanol production is evident across various pH levels. Notably, the pH adjustments from pH 4 to 9 reveal distinct impacts on the bioethanol yield. The data suggests a complex interplay

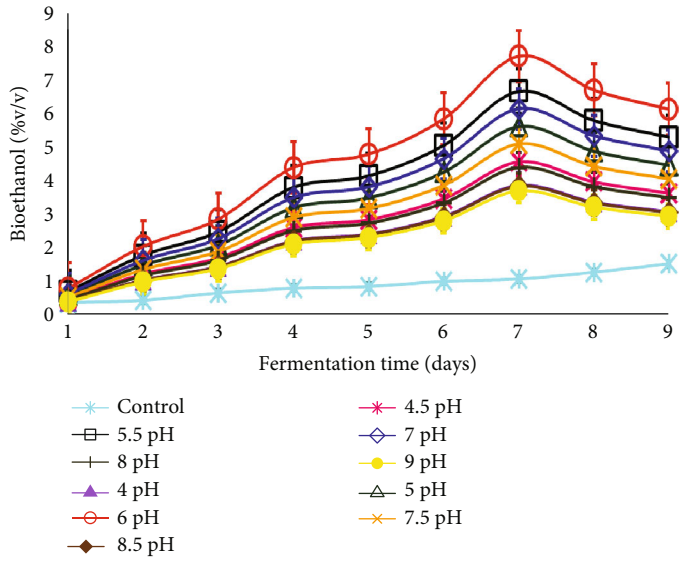

Figure 3: The influence of pH on bioethanol generation from hydrolysate POME. Other factors were fixed: 100 mL hydrolysate POME, inoculum dose 3%, temperature 30°C, agitation 150 rpm, substrate loading 5%, and fermentation time 7 days.

between fermentation time and pH, with certain pH levels demonstrating more favorable conditions for bioethanol synthesis. Understanding these trends is crucial for optimizing bioethanol production processes, as it appears that both the duration of fermentation and the acidity or alkalinity of the medium significantly influence the final ethanol output. Further analysis may unveil specific time and pH conditions that yield optimal bioethanol percentages, contributing to the advancement of biofuel production technologies.

Considering that extremely high or low pH levels in the growth medium can hinder microbial growth, maintaining a suitable pH is crucial for optimal bioethanol production. In the case of hydrolyzed POME, a pH of 6 was found to be more favorable for bioethanol production. Previous studies have also investigated the effect of pH on bioethanol production using *S. cerevisiae*. Elgharbawy et al. [23] observed the maximum bioethanol production at pH 5, while another study focusing on bioethanol production from mahua flowers found pH 6 to be the optimal condition [25]. Additionally, several researchers have reported that the optimum pH range for bioethanol production lies between 5 and 8 [22]. It is important to note that the specific pH requirement may vary depending on the substrate, production technique, and strain used. Different microorganisms may have different pH preferences, and the characteristics of the substrate being utilized can also influence the optimal pH range. Therefore, it is essential to consider the specific conditions and requirements of the system when determining the ideal initial pH for bioethanol production.

Acid pretreatment typically hydrolyzes hemicellulose and breaks down lignin, making cellulose more accessible for enzymatic hydrolysis into fermentable sugars like glucose and xylose. This can enhance the yield of reducing sugars available for fermentation, thereby increasing ethanol production. On the other hand, alkaline pretreatment is aimed at solubilizing lignin and removing hemicellulose, altering the structure of cellulose to facilitate enzymatic digestion [3, 21]. However, excessive alkalinity can lead to degradation of sugars and inhibitors that affect subsequent fermentation efficiency. The choice between acid and alkaline pretreatments depends on the specific characteristics of the biomass and the desired outcomes in terms of reducing sugar yield and ethanol production efficiency. Optimizing these pretreatment conditions is crucial for maximizing the bioethanol yield from biomass sources like POME [21].

3.2.2. Effect of Inoculum Dose. The dosage of the starter culture plays a crucial role in determining bioethanol production. A relatively low inoculation rate leads to a slower development of bacteria, resulting in less efficient consumption of the lignocellulosic materials in the fermentation media. This delay in bacterial growth can extend the time required to achieve optimal biomass and bioethanol production. On the other hand, a significant increase in the cell suspension can disrupt the linear relationship between fungal development and bioethanol production, allowing for faster and more efficient bioethanol production within a shorter time frame. However, it is worth noting that in some cases, the inoculum dosage only affects bacterial growth and does not have a significant impact on biofuel production [20].

To investigate the influence of inoculum dosage on bioethanol production, mycelial dosage was systematically increased from 1% to 7%, as depicted in Figure 4. The graph provides a comparative analysis of fermentation outcomes under different inoculum percentages and varying fermentation durations. It illustrates that an increment in the inoculum dosage from 1% to 3% resulted in higher bioethanol production. A noticeable trend emerges, indicating that higher inoculum percentages generally lead to increased fermentation results. The highest bioethanol production was observed at a 3% inoculum dosage, reaching 7.78% (*v*/*v*). Interestingly, inoculum dosages of 4% to 6% (*v*/*v*) yielded

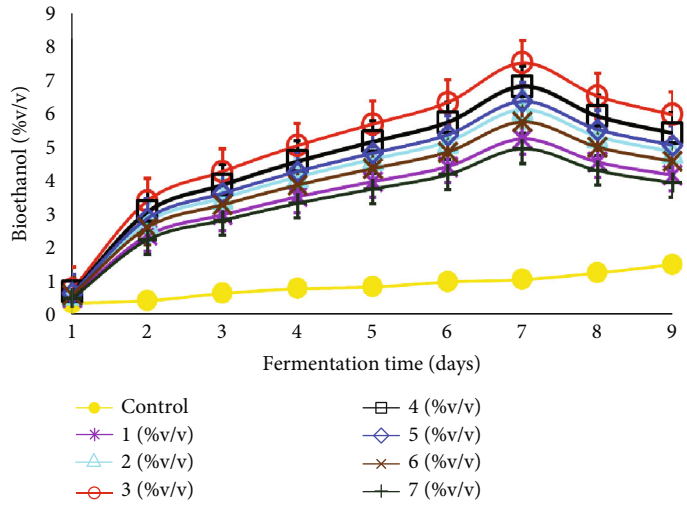

FIGURE 4: The influence of inoculum dose on bioethanol generation from hydrolysate POME. Other factors were fixed: 100 mL hydrolysate POME, pH 7, temperature 30°C, agitation 150 rpm, substrate loading 5%, and fermentation time 7 days.

nearly identical bioethanol production, while a 2% inoculum dosage led to slightly higher production. This comparative assessment underscores the dynamic nature of the relationship between inoculum concentration and fermentation outcomes. Importantly, the impact is time-dependent, with variations in the effectiveness of different inoculum concentrations observed at each fermentation interval. The data suggests a nuanced pattern where the initial increase in inoculum concentration yields significant improvements, but there may be a point of diminishing returns as concentrations continue to rise. A comprehensive understanding of this relationship necessitates further statistical analysis and visualization techniques to elucidate specific trends and identify optimal conditions for the fermentation process.

The optimal dosage of the inoculum in bioethanol production has been investigated in several studies. Alam, Kabbashi, and Hussin [22] found that a 3% inoculum dose resulted in maximum bioethanol production using *S. cerevisiae* in yeast malt extract medium (YM) broth. Similarly, another study conducted by Pandey et al. in 2016 achieved maximum bioethanol production using a 3% inoculum dose with *S. cerevisiae* (strain NCIM 3288) in YEPD broth. In contrast, Jafari Olia, Azin, and Moazami [26] utilized an inoculum size ranging from 1% to 5% to achieve optimum bioethanol production with *S. cerevisiae*. These studies demonstrate that the ideal inoculum dose for bioethanol production can vary depending on the specific strain of *S. cerevisiae*, the composition of the growth medium, and other experimental conditions. It is essential to optimize the inoculum dosage to strike a balance between promoting microbial growth and maximizing the bioethanol yield. By determining the appropriate inoculum dose based on the specific requirements of the bioethanol production process, it is possible to enhance fermentation efficiency and achieve higher bioethanol production levels.

3.2.3. Effect of Temperature. The fermentation temperature plays a pivotal role in the production of bioethanol from hydrolysate of POME using *S. cerevisiae*. Optimal fermentation conditions are vital for the metabolic activity of the yeast, with a recommended temperature range typically falling between 25°C and 35°C. This range ensures efficient yeast growth and fermentation, ultimately influencing the bioethanol yield. Higher temperatures may accelerate the fermentation process, but they also pose the risk of increased metabolic byproducts and potential contamination. Conversely, temperatures below the optimum range may slow down yeast metabolism, affecting ethanol production. Striking the right balance between productivity and yield requires careful consideration and experimentation. Factors such as enzymatic activity, substrate composition, and overall process optimization contribute to the intricate interplay of variables that define the success of bioethanol production from POME hydrolysate. Therefore, maintaining the appropriate fermentation temperature is a critical aspect of ensuring the efficiency and quality of the bioethanol production process.

Figure 5 appears to represent the bioethanol yield at different temperatures and fermentation times. It seems to be a tabular representation where the *x*-axis indicates the fermentation time (in days) and the *y*-axis represents different temperatures (in degree Celsius) at which the fermentation process was conducted. As the temperature increases, there is generally an increase in the bioethanol yield, evident by comparing values across a specific fermentation time; higher temperatures result in higher bioethanol production. An optimal temperature range for bioethanol production is identified, maximizing the yield around 7.83 (% *v*/*v*) to 8.55 (% *v*/*v*) at a fermentation incubation time of 7 days, with other factors fixed at pH 6, 150 rpm agitation, and inoculum dose at 3 (% *v*/*v*). This optimal range appears to be around 30°C–35°C, where bioethanol production is relatively high across different fermentation times. As fermentation time increases, the bioethanol yield tends to rise, particularly when comparing values in a specific temperature column across different fermentation days. Temperature sensitivity

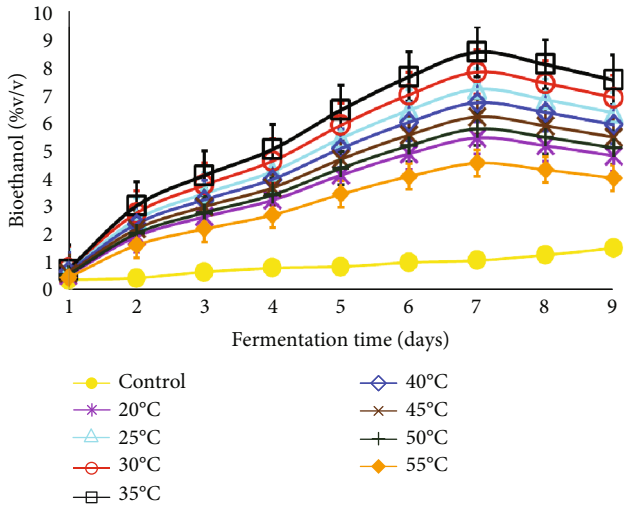

Figure 5: The influence of different temperatures on bioethanol generation from hydrolysate POME. Other factors were fixed: 100 mL hydrolysate POME, pH 7, inoculum dose 3%, agitation 150 rpm, substrate loading 5%, and fermentation time 7 days.

is observed, with extremely high temperatures (e.g., 50°C and 55°C) leading to a decrease in the bioethanol yield, especially as fermentation progresses. Based on Figure 5, optimal conditions for bioethanol production may be around 30°C–35°C with a fermentation time of 6–7 days. The lowest recorded value of 1.05, observed in the "control" at 7 days, underscores the baseline conditions, while the dataset's pinnacle, marked by a value of 8.55, occurs at 35°C after 7 days of fermentation.

This higher value at an elevated temperature suggests a potentially accelerated or more robust fermentation process under these specific conditions [17]. The contrast between the lowest and highest values within the dataset provides insights into the temperature-dependent dynamics of the fermentation process, hinting at the influence of environmental factors on the observed outcomes [27]. Temperature stands as a paramount environmental element impacting microbial functions profoundly. In the quest to pinpoint the ideal temperature for ethanol fermentation, the fermentation medium was maintained at 30°C, 35°C, and 40°C, while sustaining an initial reducing sugar concentration of 6% and a pH level of 5.5 [5, 10].

3.2.4. Effect of Fermentation Agitation. Fermentation agitation significantly influences the bioethanol production process by impacting key factors crucial to microbial activity and ethanol yields. The thorough mixing facilitated by agitation ensures a uniform distribution of oxygen, essential for the initial growth phase of microorganisms. However, as ethanol production commences, minimizing oxygen exposure becomes imperative. Agitation also plays a vital role in maintaining a consistent temperature throughout the fermentation vessel, preventing fluctuations that could adversely affect microbial metabolism. Additionally, it promotes the even distribution of nutrients, preventing local depletion or accumulation and creating an optimal environment for microbial growth. The prevention of settling, efficient mass transfer, and the suspension of yeast cells are further benefits of agitation, collectively contributing to improved fermentation efficiency and enhanced bioethanol yields. Careful optimization of agitation parameters is essential to fine-tune the process for maximum productivity in bioethanol production systems. The optimization of fermentation agitation in bioethanol production is imperative to enhance overall efficiency and productivity. By carefully fine-tuning parameters such as mixing speed and duration, this process ensures maximum exposure of microorganisms to optimal growth conditions, leading to the maximization of ethanol yields. Efficient resource utilization is another key benefit, as optimization guarantees the uniform distribution of nutrients and prevents local depletion, ultimately contributing to improved fermentation performance. Consistency in product quality is maintained through uniform mixing and temperature distribution, crucial for industries requiring precise ethanol concentrations. Moreover, by preventing microbial stress and promoting a stable fermentation environment, optimization reduces the risk of process disruptions, enhancing the reliability and sustainability of bioethanol production. This strategic approach not only meets regulatory standards but also reduces production costs, making bioethanol production processes more competitive and environmentally sustainable.

Figure 6 represents the results of an experiment investigating the impact of different agitation speeds on bioethanol production from hydrolysate POME. Bioethanol production is measured as a percentage by volume (% *v*/*v*) over a fermentation period of 9 days.

Bioethanol production generally increases over the fermentation period for all agitation speeds. This suggests ongoing fermentation activity and the conversion of sugars into ethanol by microorganisms. Higher agitation speeds generally lead to increased bioethanol production. This is evident by comparing the bioethanol yields at different agitation speeds across each fermentation day. Notably, there seems to be an optimal agitation speed where bioethanol production peaks before plateauing or slightly decreasing at higher speeds. Bioethanol production under agitation conditions (50–400 rpm) consistently exceeds that of the control (0 rpm), indicating that agitation enhances bioethanol production compared to natural fermentation. Peak bioethanol yields are observed at specific agitation speeds across different fermentation times. For instance, on Day 7, the highest bioethanol yield of 6.75% *v*/*v* is achieved at 150 rpm. There is variability in the rate of increase in bioethanol production with increasing agitation speed. While some agitation speeds show a linear increase in bioethanol yield, others exhibit a more gradual rise, suggesting different kinetic effects. Interestingly, there are fluctuations in bioethanol production at certain agitation speeds across fermentation days. For instance, at 200 rpm, bioethanol production peaks at Day 4 (4.28% *v*/*v*) before decreasing slightly on subsequent days. The data highlights the significant influence of agitation on bioethanol production from hydrolysate POME. Optimizing agitation speed is crucial for maximizing bioethanol yields during fermentation.

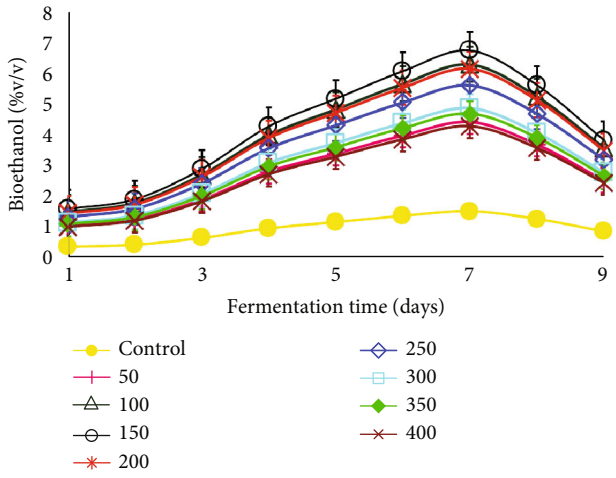

Figure 6: The influence of different agitation speeds on bioethanol production from hydrolysate POME. Other factors were fixed: 100 mL hydrolysate POME, pH 7, inoculum dose 3%, temperature 35°C, substrate loading 5%, and fermentation time 7 days.

Further studies could explore the underlying mechanisms driving the observed trends and investigate the scalability of these findings for industrial bioethanol production processes.

The ethanol production, yield, and efficiency exhibited a continual rise from the first to the ninth day of incubation under optimized conditions, as depicted in Figure 6. However, a slight decline in these parameters was noted beyond a fermentation period of 7 days. The peak ethanol yield and fermentation efficiency of 7.5 (% *v*/*v*) were achieved after 150 rpm of agitation and 7 days of incubation. Varize et al. [28] achieved a maximum fermentation efficiency of 16% from 33% of the total reducing sugars in sugarcane molasses. Similarly, Manikandan et al. [29] reported a maximum fermentation efficiency of 22.9 g/L after 48 h using wheat bran starch with *S. cerevisiae*. However, Mueansichai et al. [30] reported the highest ethanol yield and fermentation efficiency of 25.02 and 33.24 g/L, respectively, after 5 days of incubation at 30°C using *S. cerevisiae*.

3.2.5. Effect of Fermentation Substrate Loading. The efficiency of bioethanol production is intricately linked to the careful consideration of fermentation substrate loading. This parameter, defining the number of sugars or starches provided for microbial conversion, plays a pivotal role in shaping the overall process. Elevated substrate loading has the potential to boost ethanol yields and fermentation productivity, resulting in shorter fermentation times. However, finding the optimum loading level is crucial, as excessively high concentrations may hinder microbial growth due to osmotic stress and lead to increased ethanol concentrations, acting as inhibitors. Balancing these factors is essential for maintaining microbial viability, preventing contamination, and ensuring the economic sustainability of the process. Additionally, the impact of substrate loading must be carefully assessed at different scales of production, considering the dynamics of waste generation and the overall efficiency of the bioethanol production system. In essence, optimizing substrate loading is a key aspect of designing a successful and economically viable bioethanol production process.

Figure 7 shows the influence of different substrate loadings on the production of bioethanol from hydrolysate POME over a period of 9 days. At the beginning of the fermentation process (Day 1), the bioethanol production ranges from 0.35% *v*/*v* for the control to 1.77% *v*/*v* for the highest substrate loading of 5.5% *v*/*v*. As fermentation progresses, bioethanol production generally increases across all substrate loadings. For example, by Day 3, bioethanol production ranges from 0.64% *v*/*v* for the control to 3.23% *v*/*v* for the highest substrate loading. Comparing bioethanol production at each fermentation time across different substrate loadings, it is evident that higher substrate loadings result in higher bioethanol yields. For instance, on Day 6, bioethanol production ranges from 1.35% *v*/*v* for the control to 6.82% *v*/*v* for the highest substrate loading. While higher substrate loadings generally lead to higher bioethanol production, there may be diminishing returns observed at later fermentation times. For example, on Day 9, the bioethanol production ranges from 1.2% *v*/*v* for the control to 6.06% *v*/*v* for the highest substrate loading, showing smaller incremental increases compared to earlier fermentation times. Figure 7 suggests that there may be an optimal substrate loading that maximizes bioethanol production. Further analysis could focus on identifying this optimal point to improve the efficiency of bioethanol production processes. It is important to acknowledge that factors beyond substrate loading, such as pH, temperature, and the composition of the fermentation medium, can also influence bioethanol production [21].

Understanding the interactions between these factors is crucial for optimizing bioethanol production. The data provides valuable insights for scaling up bioethanol production from hydrolysate POME [13]. By understanding the relationship between substrate loading and bioethanol yield, researchers and industry professionals can design more efficient fermentation processes for larger-scale production [31].

3.2.6. Effect of Fermentation Time. The effect of incubation time on bioethanol production was investigated by monitoring the amount of bioethanol generated at 24-h intervals up to 168 h. As shown in Figure 8, the incubation period had a significant impact on bioethanol production, with the highest production observed after 3 days (72 h) of incubation time at a concentration of 6.75% *v*/*v*. Prolonged fermentation beyond this optimal time resulted in a decrease in bioethanol production, reaching 4.79% *v*/*v* after 7 days (168 h) of culture. Although the concentration of cellulosic ethanol started to decline after 4 days of fermentation, the optimization technique maintained a fermentation period of 3 days. This finding is consistent with the study conducted by Alam, Kabbashi, and Hussin [22], where a fermentation time of 3 days was utilized to achieve a bioethanol yield of 4% *v*/*v* from *Trichoderma harzianum* and *S. cerevisiae*.

These results suggest that an optimal incubation time exists for bioethanol production, beyond which prolonged

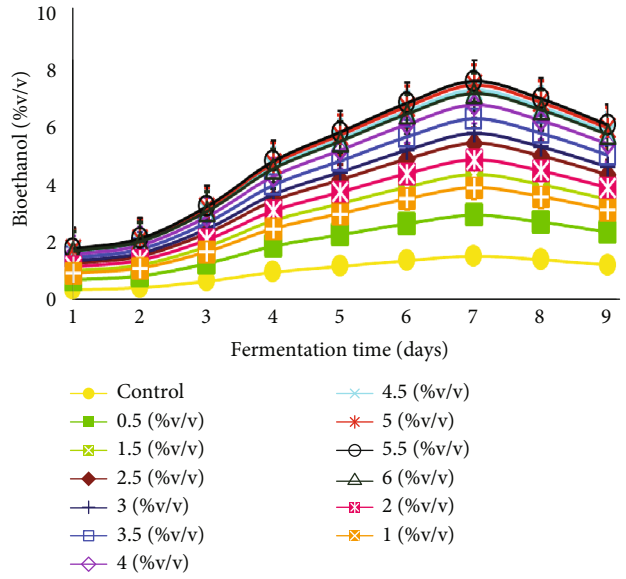

FIGURE 7: The influence of different substrate loadings on bioethanol production from hydrolysate POME. Other factors were fixed: 100 mL hydrolysate POME, pH 7, inoculum dose 3%, temperature 35°C, agitation 150 rpm, and fermentation time 7 days.

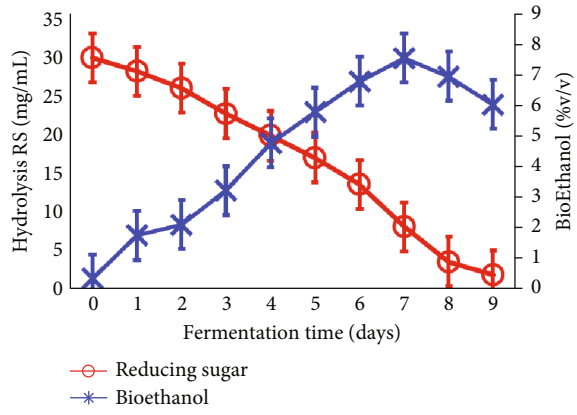

FIGURE 8: The influence of fermentation time on bioethanol generation from hydrolyzed reducing sugar. Other factors were fixed: 100 mL hydrolysate POME, pH 7, inoculum dose 3%, temperature 35°C, agitation 150 rpm, and substrate loading 5%.

fermentation does not yield significant increases in bioethanol concentration. By carefully selecting and controlling the fermentation period, it is possible to maximize bioethanol production efficiency and achieve desirable ethanol concentrations within a shorter time frame. Mueansichai et al. [30] reported a maximum ethanol yield of 0.18 g/L after 36 h of incubation time, while another study [29] demonstrated the highest ethanol yield of 24.8 g/L after 48 h of incubation time using *S. cerevisiae* strains (baker's yeast and distiller's yeast). These findings highlight the variation in optimal incubation times and ethanol yields depending on the specific yeast strain and experimental conditions employed. Although the OFAT method has been commonly used to develop bioethanol production processes, it does have certain limitations. It is time-consuming and labor-intensive and fails to consider the potential interactions between different factors. To overcome these limitations, an optimization method using experimental design, such as the FCCCD, was employed in the subsequent experiments. The FCCCD approach allows for the efficient exploration of the experimental space, considering the interactions between variables, and provides a systematic approach to achieving optimal bioethanol production.

3.3. Statistical Optimization of the Fermentation Process by FCCCD Under RSM. In the context of RSM and FCCCD for optimizing fermentation processes, the selection of pH, inoculum dosage, and reducing sugar content as criteria is strategic. pH directly influences enzyme activity and microbial growth, crucial for fermentation efficiency. Inoculum dosage affects initial microbial population and fermentation kinetics. Reducing sugar content serves as the substrate for microbial metabolism, impacting the product yield. By varying these factors within a designed experiment, RSM is aimed at understanding their combined effects and identifying optimal conditions for maximizing bioethanol production or other fermentation outputs efficiently. This systematic approach helps in achieving robust process optimization by balancing these key variables to enhance fermentation performance. To overcome the limitations of single-factor analysis, an optimization method called FCCCD was employed. This method is aimed at identifying the optimum conditions and evaluating the factors that contribute to the maximum response [24]. By considering the interactions and quadratic and linear effects of various treatments, bioconversion can be optimized. Two commonly used statistical optimization methods are RSM and fractional factorial design. In this study, the FCCCD experimental design was used to improve three independent variables: inoculum dose, pH, and reducing sugar content, for bioethanol production using hydrolyzed POME as the basal medium. By incorporating significant findings from the initial OFAT investigations, the FCCCD approach allowed for the evaluation of interactions between variables. The main objective of this optimized technique was to develop a statistical method that provides a better understanding of the parameter interactions in bioethanol synthesis, while reducing the complexity and cost of experiments.

In the FCCCD design, the decision to include pH ranging from 4 to 8 and inoculum dosage ranging from 2% to 4% *v/v* is based on their known significant impacts on fermentation processes. pH influences enzyme activity and microbial growth, crucial for fermentation efficiency, while inoculum dosage affects initial microbial population and fermentation kinetics. However, despite reducing sugar content not traditionally considered influential in ethanol production, its inclusion within the range of 15%–25% mg/mL allows for a comprehensive exploration of potential effects on fermentation outcomes. This approach ensures a thorough investigation of all relevant factors affecting the fermentation process, even if not initially identified as influential in Table 1 of the study's design considerations.

Table 3: Observed and predicted values of FCCCD variables and bioethanol production.

Run	Factor 1 *A* : pH	Factor 2 *B* : inoculum dose % *v*/*v*	Factor 3 *C* : reducing sugar in hydrolysate % mg/mL	Response 1 Bioethanol (actual) % *v*/*v*	Response 1 Bioethanol (predicted) % *v*/*v*
1	6	2	20	6.84	6.82
2	8	2	15	4.52	4.53
3	6	4	20	6.35	6.34
4	6	3	25	7.64	7.63
5	6	3	15	6.43	6.41
6	8	2	25	5.75	5.76
7	8	4	15	3.45	3.47
8	**6**	**3**	**20**	**6.99**	**7.12**
9	8	3	20	5.47	5.42
10	4	2	25	6.27	6.26
11	4	4	15	5.15	5.14
12	4	3	20	6.48	6.50
13	**6**	**3**	**20**	**7.29**	**7.12**
14	8	4	25	5.39	5.40
15	4	2	15	5.74	5.74
16	**6**	**3**	**20**	**7.17**	**7.12**
17	**6**	**3**	**20**	**7.11**	**7.12**
18	4	4	25	6.37	6.36
19	**6**	**3**	**20**	**7.07**	**7.12**
20	**6**	**3**	**20**	**7.01**	**7.12**

Note: Bold indicates center points.

Other components that had smaller fluctuations in bioethanol synthesis were fixed based on the important factors identified through OFAT. Table 3 summarizes the observed and predicted values of the bioethanol yield for each experimental run, based on the regression equation derived from the 20 runs. The highest bioethanol production of 7.64% (*v*/*v*) was achieved in Run 4, while the lowest bioethanol production of 3.45% (*v*/*v*) was observed in Run 7.

The regression coefficients of the equation were calculated using polynomial regression analysis based on the experimental observations. This formed equation was then utilized to estimate the bioethanol yield. By applying this regression model, the bioethanol production can be predicted for various combinations of the independent variables in order to optimize the bioethanol synthesis process. The experimental values represent the actual bioethanol production obtained from each run, while the predicted values were calculated using the regression equation developed based on the experimental data.

A comparison between the experimental and predicted values provides an assessment of the accuracy of the regression model in estimating bioethanol production. The mathematical model equation, obtained through polynomial statistical analysis, represents the relationship between bioethanol yields and the coded components of pH, inoculum dosage, and reducing sugar concentration. This equation, denoted as Equation (1), allows for the expression of bioethanol yields in terms of these variables.

$$Y = +7.12 - 0.54 \times A - 0.24 \times B + 0.61 \times C - 0.12 \times AB + 0.18 \times AC + 0.18 \times BC - 1.15 \times A^2 - 0.53 \times B^2 - 0.094 \times C^2 \quad (7)$$

where Y is the bioethanol production (% *v*/*v*), A is the pH, B is the inoculum dose (% *v*/*v*), and C is the reducing sugar content (% mg/mL).

The appropriateness of the predictive model was evaluated using Fischer's regression equation and ANOVA. The results of the ANOVA statistics are presented in Table 4. The F value of 340.58 and a p value of 0.0001 indicate that the polynomial model recommended is statistically significant. The terms A, B, AB, AC, BC, A^2, B^2, and C^2 were found to be significant model parameters that have a significant impact on total bioethanol production. Among these variables, the reducing sugar concentration exhibited the highest impact on bioethanol generation, while the inoculum had the shortest significant impact. The lack-of-fit F value of 0.089 suggests that the lack of fit is not significant compared to the concentrated error, indicating that the model is a good fit for the data. The coefficient of determination (R^2) value near one indicates a strong relationship between the actual and predicted values.

In this case, the model demonstrated a high level of effectiveness with an R^2 value of 0.9967 and adjusted R^2 value of 0.9938. The signal-to-noise ratio, which assesses

Table 4: ANOVA conducted based on the experimental runs of the FCCCD to assess the significance of factors influencing bioethanol production.

Source	Sum of squares	df	Mean square	*F* value	*p* value Prob > *F*	
Model	20.68	9	2.30	340.58	< 0.0001	Significant
A—pH	2.95	1	2.95	437.12	< 0.0001	
B—Inoculum	0.58	1	0.58	86.11	< 0.0001	
C—Sugar content	3.76	1	3.76	557.08	< 0.0001	
AB	0.11	1	0.11	16.37	0.0023	
AC	0.25	1	0.25	37.37	0.0001	
BC	0.25	1	0.25	36.32	0.0001	
A^2	3.66	1	3.66	543.02	< 0.0001	
B^2	0.78	1	0.78	116.30	< 0.0001	
C^2	0.024	1	0.024	3.61	0.0866	
Residual	0.067	10	6.745e – 003			
Lack of fit	5.519e – 003	5	1.104e – 003	0.089	0.9905	Not significant
Pure error	0.062	5	0.012			
Cor total	20.74	19				

Note: $R^2 = 0.9967$, adjusted $R^2 = 0.9938$, CV = 1.32, predicted $R^2 = 0.9943$, and adequate precision = 71.77.

the model's accuracy, was evaluated and found to be 71.773, indicating a strong model with acceptable accuracy (a ratio above 4). On the other hand, the coefficient of variation (CV) provides insights into the dispersion of the obtained observations. In the case of bioethanol production, the CV was calculated to be 1.32%, indicating a relatively low variation and improved repeatability. A lower CV value, closer to zero, is desirable for a response design, as it suggests a more consistent and reliable outcome [32].

Figures 9(a) and 9(b) illustrate the relationship between pH and inoculum dosage on bioethanol production when the reducing sugar concentration is set at the center value.

Both parameters exhibited exponential effects on bioethanol generation, resulting in an elliptical-shaped response plot. These findings highlight the significance of optimizing pH and inoculum dosage to achieve optimum performance conditions. Further optimization beyond these levels led to a decline in bioethanol production. These parameters are crucial for bioethanol synthesis and are considered key indicators of efficiency and economic impact. Exceeding their optimal values can result in unnecessary costs and a decrease in bioethanol production.

Figures 10(a) and 10(b) provide insights into the effect of reducing sugar content and pH on bioethanol synthesis, with the inoculum dosage held constant at the center value. The 3D and 2D surface curves in these figures visually represent the relationship and correlation between the evaluated factors.

The distinct shapes and patterns observed in the curves indicate a significant influence and interaction between reducing sugar content and pH on bioethanol production. These graphical representations help in understanding the complex dynamics and optimizing the conditions for maximizing bioethanol synthesis. The influence of pH on bioethanol synthesis exhibited a quadratic relationship. As the pH of the solution increased up to approximately pH 6, bioethanol production showed an increasing trend. However, beyond this pH value, the bioethanol synthesis started to decline. On the other hand, reducing the sugar concentration had a linear effect on the bioethanol yield. Lowering the sugar content resulted in an increase in bioethanol production without significant deviations within the pH range studied in this investigation.

Figures 11(a) and 11(b) illustrate the interaction between reducing sugar content and inoculum dose at a constant pH of 6. The results show that increasing the concentrations of reducing sugar and inoculum dose does not result in an increase in bioethanol production. The impact of inoculum dose on bioethanol production was computationally efficient, meaning that it had a noticeable effect on the yield. However, the influence of reducing sugar content on bioethanol production was exponential and independent of the fraction of inoculum dose in the medium. This suggests that manipulating the reducing sugar content had a more significant impact on bioethanol production compared to the inoculum dose. Inoculum dosage formulations closer to the reduced operating points of the variables showed a stronger response compared to those closer to the significantly increased development perspective of the variables. This finding is consistent with the results reported by Zhai, Hu, and Jin [33] and Rashid and Alam [32] in their studies on optimizing bioethanol production using RSM. They found that the optimum bioethanol production was achieved at moderate levels of reducing sugar content and pH.

Two commonly used methods for systematically analyzing the target factors in optimization studies are central composite design (CCD) and Plackett–Burman design (PBD). PBD is particularly effective as a screening design, as it significantly reduces the number of experiments while

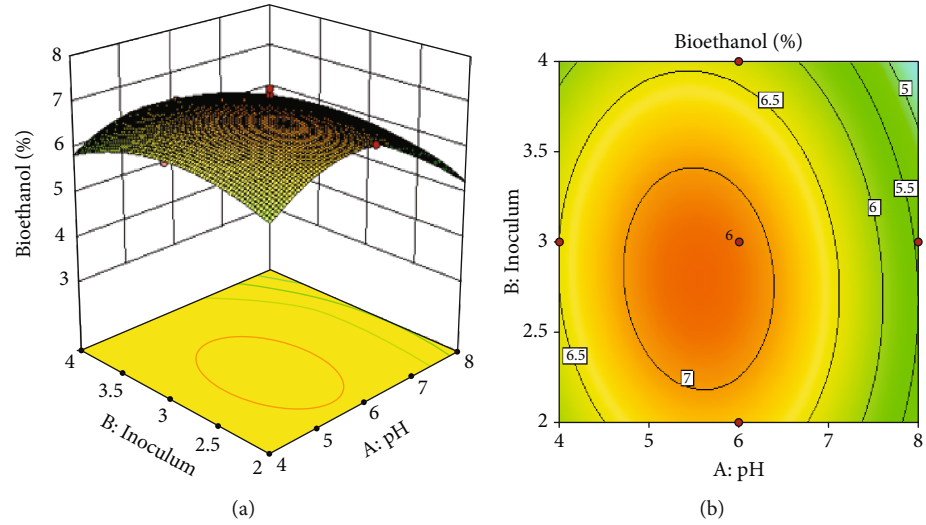

FIGURE 9: The connectivity influences of (*A*) pH and (*B*) inoculum dosage were illustrated in the (a) 3D response surface curve and (b) 2D contour plot.

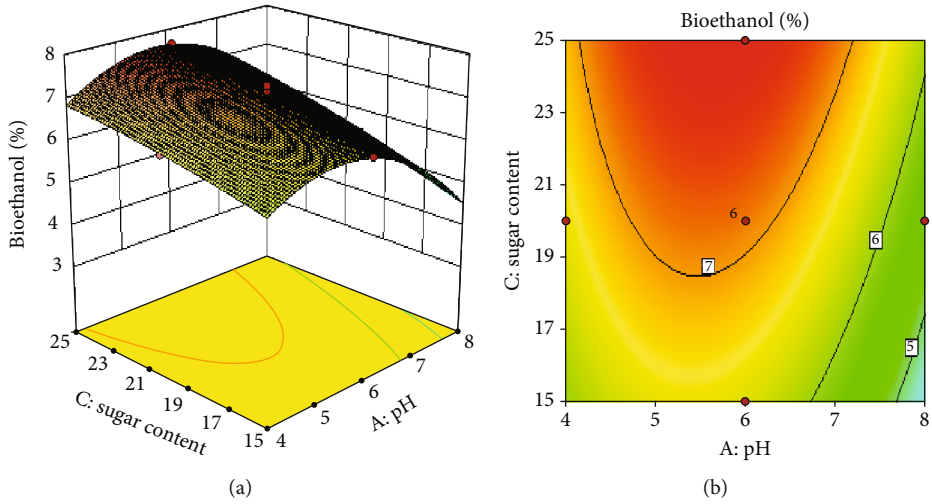

FIGURE 10: The connectivity influences of (*A*) pH and (*C*) reducing sugar content were illustrated in the (a) 3D response surface curve and (b) contour plot.

still providing valuable information for assessing the target factors. Only the most significant factors are selected for further optimization studies, while those with less impact on the response value may be excluded from further experimentation.

PBD has been widely used in various fields, such as formulating multicomponent systems and media optimization. The selected parameters, narrowed down through PBD, are then optimized using CCD, which takes into account quadratic, interaction, and linear effects in the treatment. Many researchers have successfully applied these optimization methods to maximize bioethanol production [22].

3.4. An Experimental Model's Verification. The validity and reliability of the interface model were confirmed through a statistical approach. To assess the accuracy of the model's predictions, experimental data were collected and compared to the predicted values using Design-Expert software Version 1. The predictions were performed in triplicate, evaluating three different configurations of medium ingredients for predicting bioethanol synthesis. The results were then analyzed by comparing the experimental response to the expected values, as shown in Table 5. The comparison revealed that the experimental response was very close to the expected values, with an error percentage of only 7%. This high level of agreement between the experimental and predicted values further strengthens the reliability and suitability of the projected predictions. The optimization study resulted in significant improvements in bioethanol production compared to the traditional OFAT approach.

By utilizing the optimum experimental conditions obtained through FCCCD, the bioethanol production increased to 7.64% (*v*/*v*), as depicted in Figure 12. This represents a substantial increase in the bioethanol yield compared

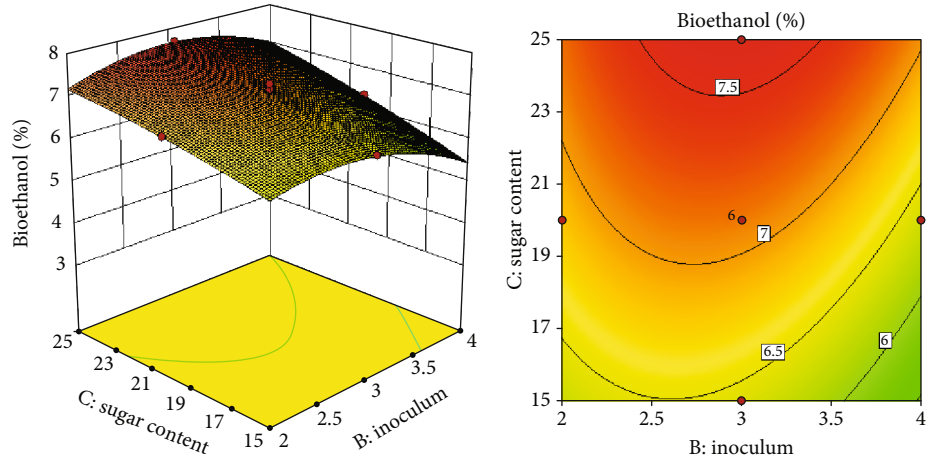

Figure 11: The connectivity influences of (*B*) inoculum dose and (*C*) reducing sugar content were illustrated in the (a) 3D response surface curve and (b) contour plot.

Table 5: An experimental model verification for higher bioethanol production.

Experiment	pH	Inoculum (% *v*/*v*)	RS (% g/L)	Bioethanol (% *v*/*v*) (theoretical)	Bioethanol (*v*/*v*) (experimental)	Error (%)
1	4.5	17	3	6.55	6.35	+3.14
2	5	20	3.5	6.87	6.94	−1.01
3	7	15	2.5	5.94	5.53	+6.9

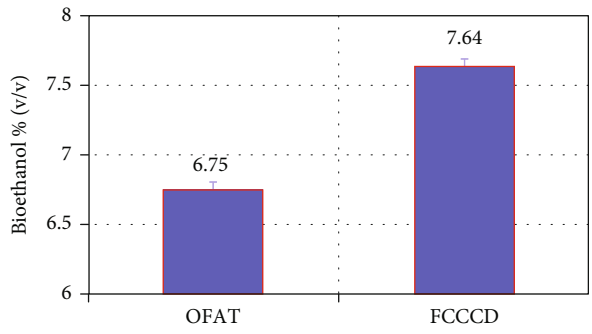

Figure 12: Graphic of biological conversion enhancement from OFAT to FCCCD.

to the results obtained through the OFAT method. Additionally, the fermentation time was significantly reduced to 72 h, a significant improvement over the 7 days (168 h) required in the OFAT experiments. These findings highlight the effectiveness of the optimization approach in enhancing bioethanol production efficiency and reducing the time required for fermentation.

The study yielded a highly successful and significant improvement in bioethanol production. The findings indicated that the factors of pH, inoculum dose, and reducing sugar content played crucial roles in achieving high bioethanol yields. These three variables were identified as the key factors influencing bioethanol production during the study. By optimizing these factors, the study was able to achieve notable enhancements in bioethanol synthesis. The results highlight the importance of understanding and controlling these specific parameters to maximize bioethanol production efficiency. To confirm the presence of bioethanol, GC/MS analysis was conducted. The results indicated that the mass spectrum (MS) fraction of bioethanol was 6.07 as depicted in Figure S2. This MS fraction closely resembled the standard MS fraction of 14:19:27:29 (S2), providing further evidence of the formation of bioethanol.

3.5. Kinetic Study of the Fermentation Process. During the kinetic study, the interaction of bioethanol production and total sugar consumption and biomass growth were observed from the fermentation medium as shown in Figure 13. Biomass was determined as a growth indicator of fungal growth as it was a significant and steady component in mycelial cell walls. The biomass content appears to be a suitable factor in the approximation of the total sum of the bioethanol production, and its modifications may parallel the development of biomass growth. While the bioethanol production was increased and also the biomass growth increased, the reducing sugar decreased [28]. Figure 10 shows the result of the growth kinetic study. It was presented that the consumption by reducing sugar decreased sharply with the sharp rise of bioethanol together with the biomass growth, in terms of bioethanol, until the product reaches its highest level, and after that, all three parameters progress slowly. The reducing sugar content in the fermentation broth reduced to 3.2 mg/mL from the initial value of 11.21 mg/mL after 3 days of fermentation, and the bioethanol production level was at 3.12 (% *v*/*v*) when the bioethanol production attained the highest

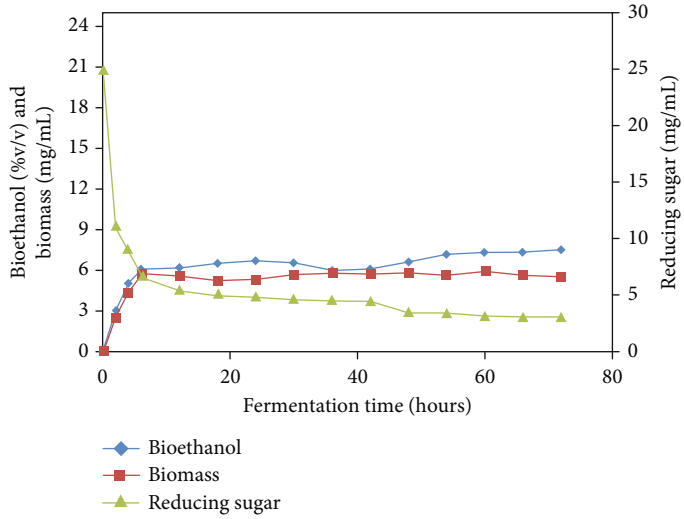

Figure 13: Final bioethanol production, biomass growth, and sugar consumption trends after 72 h of fermentation.

level (7.53% *v*/*v*). Through the progress of fermentation, the reduced sugar content becomes less and the value was 3.39 mg/mL after 60 h of fermentation. The biomass growth increased from 60 h, and after that, it sharply declined to 5.55% (mg/mL) after 3 days of fermentation.

The relationship of the C-source consumption, biomass growth, and bioethanol production that was obtained during the production of bioethanol by *S. cerevisiae* through LSF of POME was found to be in agreement with the classical kinetic model of this type. It was possible to compute the particular rate of product creation. The real production of product from biomass growth was Y_p/x, while the specific rate of product creation related to preservation was m_p. The first term was growth associated, and the second term was nongrowth associated which can be neglected in this study where product formation did not occur due to maintenance. Finally, q_p was the product of the theoretical yield of product from biomass Y_p/x (1.208 g g^{-1}) and a specific rate of growth μ (0.198 h^{-1}). The maximum specific rate of product formation was estimated to be about 0.239 h^{-1}.

Whereas the yield of bioethanol production based on total reducing sugar consumption Y_p/s is 0.374 g g^{-1}, the yield of biomass formation based on reducing sugar consumption Y_x/s is 0.305 g g^{-1}, from the experimental results. The model with the highest coefficient of determination (R^2) was regarded as the best fit for the enzyme. Table 6 summarizes the kinetic parameters for each linearized model. The model with the highest coefficient of determination (R^2) was regarded as the best fit for the enzyme. It was observed from Table 6 that the exponential growth occurred until 6 h of fermentation and the overall specific growth rate (μ) was estimated to be 0.198 h^{-1}.

Many researchers [28] have reported on optimum bioethanol production at a low specific growth rate (0.178 h^{-1}) using *S. cerevisiae*, and Wang and Liu [34] found the optimum bioethanol production at a lower specific growth rate (0.229 ± 0.030 h^{-1}) using *Escherichia coli* FBWHR in the hot-water sugar maple wood extract hydrolysate-based medium.

Table 6: Kinetic parameters for bioethanol production.

Parameters	Units	Value	R^2
μ	h^{-1}	0.198	0.9978
Y_p/x	g g^{-1}	1.208	0.9815
Y_p/s	g g^{-1}	0.374	0.9874
Y_x/s	g g^{-1}	0.305	0.9766

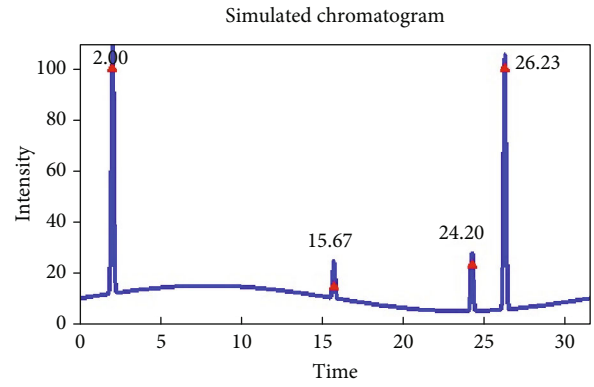

Figure 14: GC/MS analysis chromatogram for bioethanol production.

3.6. Bioethanol Production Using GC/MS. Bioethanol production using the chromic acid method and GC/MS highlights the strengths and limitations of each technique. The chromic acid method, yielding a bioethanol production of 7.64% (*v*/*v*), involves a chemical reaction that provides a rapid and cost-effective estimate of ethanol concentration. This method is suitable for preliminary screenings or situations where quick approximations are adequate. However,

Table 7: Comparative analysis and another recent works on bioethanol production.

Aspect	Proposed works	Reference [35–38]
Objective	Efficient bioethanol production from POME using SRS	Bioethanol production from POME using batch fermentation
Methodology	Acid–base pretreatment, enzymatic hydrolysis, fermentation	Enzymatic hydrolysis, batch fermentation
Pretreatment technique	Acid–base pretreatment	Steam explosion pretreatment
Enzyme source	Locally sourced cellulase enzyme	Commercial cellulase enzyme
Yield of reducing sugars (g/L)	26.6 (53.14%)	22.3 (47.1%)
Bioethanol yield (%)	OFAT: 6.75, FCCCD: 7.64	Batch fermentation: 5.8
Kinetic parameters	$\mu = 0.198\,h^{-1}$ and $q_p = 0.239\,h^{-1}$ after 3 days	$\mu = 0.155\,h^{-1}$ and $q_p = 0.190\,h^{-1}$ after 4 days
Main findings	Integrated SRS improves bioethanol production efficiency	Steam explosion enhances sugar yield and bioethanol production
Significance	Sustainable POME management, economic benefits	Viable method for large scale

it may be less precise due to potential interferences from other substances and the manual nature of the procedure. Despite its simplicity and accessibility, the chromic acid method's sensitivity can be lower, particularly in samples with low ethanol content or complex matrices. On the other hand, GC/MS, which measured bioethanol production at 7.77% (*v*/*v*), offers a higher level of accuracy and precision. This advanced analytical technique separates ethanol from other components in the sample and provides a detailed mass spectral analysis. Although more expensive and requiring skilled personnel to operate, GC/MS is ideal for confirmatory analysis, regulatory compliance, or research requiring high precision. The slight difference of 0.13% (*v*/*v*) between the two methods underscores GC/MS's superior sensitivity and ability to accurately quantify ethanol.

In the GC/MS analysis, the chromatogram for bioethanol revealed four distinct functional groups within the retention time range of 2.00 to 26.23 min (Figure 14).

The chromatogram identified ethanol, or alcohol, at a retention time of 2.60 min. Additionally, other peaks were identified at specific retention times: acetic acid/benzoic acid at 15.67 min, methyl-2-pentene at 24.20 min, and propionic acid at 26.23 min. The percentage difference of 1.70% further illustrates the close agreement between the methods while highlighting GC/MS's enhanced precision. Thus, while the chromic acid method is valuable for quick and cost-effective estimates, GC/MS stands out for its detailed and reliable quantification, making it the preferred choice for detailed analytical needs.

3.7. Comparative Analysis and Another Works. Table 7 shows that the proposed method is aimed at enhancing bioethanol production from POME by implementing a SRS that integrates acid–base pretreatment and enzymatic hydrolysis techniques. They achieved a maximum yield of reducing sugars of 26.6 g/L (53.14%) and optimized bioethanol yields of 6.75% *v*/*v* using the OFAT approach and 7.64% *v*/*v* using the FCCCD.

Kinetic analysis showed a specific growth rate (μ) of $0.198\,h^{-1}$ and a specific product formation rate (q_p) of $0.239\,h^{-1}$ after 3 days of fermentation. This approach not only streamlined the bioethanol production process but also offered economic benefits through efficient POME management. In contrast, in the reference study, Mazaheri et al. [35], Jayakumar et al. [36] and Tse, Wiens, and Reaney [37] reviewed the potential of palm oil industrial wastes, including POME, for biohydrogen production. They discussed various methods such as thermal pretreatment to enhance anaerobic digestion performance, focusing on biogas production. While their study did not specify quantitative outcomes like reducing sugar yields or bioethanol production rates, it highlighted the broader application of industrial waste streams like POME in renewable energy production. Both studies underscore the importance of sustainable waste management practices in the biofuel sector, albeit with different emphases on bioethanol and biohydrogen production pathways [38].

4. Conclusion

In this study, a single bioreactor is developed to complete the full process of bioethanol production (fermentation) including upstream (acid and base pretreatment, enzymatic hydrolysis) and downstream processing. The results showed a higher production of bioethanol (7.64% *v*/*v*) by combined effects of the OFAT and FCCCD as the optimization methods. In addition, it was found from the kinetic study of the production of the bioethanol with the development process condition that the products in this system are growth associated and the specific growth rate (μ) was estimated to be $0.198\,h^{-1}$ and the maximum specific rate of product formation was determined to be about $0.239\,h^{-1}$. The integration of acid–base pretreatment and enzymatic hydrolysis in a SRS for bioethanol production from POME presents a promising approach. This study optimized processes to achieve a significant yield of reducing sugars and bioethanol, demonstrating economic feasibility through reduced operational costs and simplified processing steps. By utilizing locally sourced enzymes and optimizing fermentation parameters, the study enhances the economic viability of POME utilization in biofuel production. Technologically, the SRS offers a streamlined method that could be scaled up

for industrial applications, contributing to sustainable practices in the palm oil industry. Future research should focus on further optimizing operational conditions and scaling up production to validate its commercial viability and broader environmental benefits.

Data Availability Statement

The data supporting this research article are available from the corresponding author or first author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. Temperature plays a critical role in microbial cell growth, directly influencing enzymatic activities and metabolic rates. Optimal temperatures enhance growth, while deviations can inhibit cell function or lead to cell death. The impact of temperature on microbial cell growth during syngas fermentation was examined, as depicted in Figure S1. The findings revealed that the highest microbial growth occurred at a temperature of 35°C. Consequently, to optimize the bioethanol yield, the entire syngas fermentation process was conducted at this optimal temperature of 35°C. GC/MS is an analytical technique used to identify and quantify chemical compounds in a sample. In the context of bioethanol production from POME, GC/MS can be used to analyze the composition of the POME, monitor the fermentation process, and determine the concentration of bioethanol in the final product. The results of the GC/MS analysis are presented in Figure S2. According to existing literature, the expected mass spectrum (MS) for ethanol is 6.65 (% *v*/*v*). In our study, the MS value obtained from the GC/MS analysis was 7 (% *v*/*v*). Analysis of the fragmentation data revealed that MS 27 corresponds to $[CH_2\text{-}OH]^+$, indicating a transformation from $[CH_2OH]^+$ to the more stable cation $[CH_3{=}O]^+$. Additionally, MS 14 corresponds to $[CH_3]^+$, while MS 29 corresponds to $[CH_3CH_2]^+$. These findings suggest that *Saccharomyces cerevisiae* facilitated the production of bioethanol from hydrogen-containing syngas. Furthermore, byproduct charcoal played a role in aiding microbial fermentation by providing carbon nutrients, trace elements, and minerals, as detected through XRF analysis (S2). Based on the preceding analysis, it is established that bioethanol was generated via fermentation employing *S. cerevisiae*. Consequently, biomass-derived liquid state fermentation proved to be suitable for bioethanol production. By utilizing GC/MS analysis, bioethanol production from POME can be optimized to improve the efficiency and quality of the bioethanol produced. *(Supporting Information)*

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