

Single-Factor Screening and Response Surface Optimization of Co-Culture Simultaneous Saccharification and Fermentation of Rice Husk (*Oryza sativa* cv. Ciherang) for Bioethanol Production

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Rice husk is an abundant agricultural residue in Indonesia and represents a promising non-food lignocellulosic feedstock for second-generation bioethanol. This study investigated the production of bioethanol from rice husk (*Oryza sativa* cv. Ciherang) through simultaneous saccharification and fermentation (SSF) using *Aspergillus niger* and *Saccharomyces cerevisiae*. Rice husk was pretreated with 2 M NaOH at ambient temperature to disrupt the lignocellulosic matrix and improve substrate accessibility prior to simultaneous saccharification and fermentation. Single-factor experiments were first conducted to evaluate the effects of fermentation time, pH, temperature, and starter concentration on ethanol yield. Response Surface Methodology (RSM) employing a Central Composite Design (CCD) with 31 experimental runs was subsequently used to optimize the SSF process. The quadratic model produced a high coefficient of determination ($R^2 = 0.9575$) and a non-significant lack-of-fit, indicating excellent model adequacy. Temperature and fermentation time were identified as the most influential factors, while quadratic effects were significant for all variables, confirming the nonlinear behavior of the system. The stationary point of the response surface was located at 98.80 h, pH 5.18, 42.38 °C and 2.07 mL of each starter, at which the ethanol concentration reached $9.08 \pm 0.63\%$ (v/v). This study demonstrates the potential of rice husk valorization through SSF and highlights the effectiveness of RSM for optimizing multivariable bioethanol processes.

Keywords: Rice husk, bioethanol, simultaneous saccharification and fermentation, response surface methodology, lignocellulosic biomass

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The global transition toward sustainable and low-carbon energy has intensified the search for renewable biofuels capable of reducing dependence on fossil resources. Bioethanol remains one of the most promising alternatives due to its high oxygen content, clean-burning properties, and compatibility as a blend or substitute for gasoline [1, 2]. However, conventional first-generation bioethanol production relies on food-based feedstocks such as corn, molasses, and sugarcane, raising concerns about food-fuel competition, rising feedstock costs, and pressure on agricultural land [3]. In response, attention has increasingly shifted toward lignocellulosic residues as substrates for second-generation bioethanol, offering a sustainable and non-competitive resource base for biofuel production [4].

Indonesia, as one of the world largest rice-producing countries, generates millions of tons of rice husk annually as a byproduct of rice milling.

Rice is the dominant staple food for over 270 million Indonesians, resulting in continuous and abundant husk production with minimal seasonal variation [5]. In 2025, produced approximately 60.21 million tonnes of dry unhusked rice. As the rice husk accounts for approximately 20-22% of the mass processed during rice milling, this production represents an estimated rice-husk potential of approximately 12.04-13.25 million tonnes [6, 7]. Despite its availability, rice husk is often underutilized, commonly disposed of through open burning, or used inefficiently as low-grade fuel. Such practices contribute to particulate emissions and environmental degradation [8]. Because rice husk is not used as a food source and possesses a high cellulose-hemicellulose fraction, its valorization into bioethanol represents a strategic pathway to support Indonesia renewable energy goals while mitigating biomass waste accumulation. Rice husk is a lignocellulosic agricultural residue containing a substantial cellulose fraction that can serve as a source

of fermentable sugars for second-generation bioethanol production. Rice husk contains approximately 34-44% cellulose, 23-30% lignin, 13-39% ash, and 8-15% moisture. The ash fraction is predominantly composed of silica, with reported SiO₂ contents of 86.90-97.30% from the total ash content [7]. Previous studies have highlighted the feasibility of converting rice husk into fermentable sugars. However, its high lignin content necessitates effective pretreatment to enhance enzymatic digestibility [4, 9].

Co-culture strategies have been investigated as an approach for integrating the hydrolysis of lignocellulosic substrates with the microbial conversion of released sugars into ethanol [10]. In an *Aspergillus niger*-*Saccharomyces cerevisiae* system, the two microorganisms perform complementary functions within the fermentation process, with the fungal component contributing to substrate hydrolysis and the yeast converting available fermentable sugars into ethanol [11]. Nevertheless, the effectiveness of such a co-culture depends on operational conditions that accommodate both microorganisms. Differences in their responses to pH, temperature, fermentation duration, and inoculum level may result in an imbalance between sugar release and sugar fermentation [12]. Although fungal-yeast co-cultures have been investigated using several lignocellulosic substrates, information regarding the systematic optimization of this co-culture for alkali-pretreated Ciherang rice husk remains limited. Therefore, the present study employed single-factor screening followed by response surface methodology to determine the operating conditions that maximize ethanol production from this locally available residue.

Simultaneous Saccharification and Fermentation (SSF) is a well-established approach for lignocellulosic bioethanol production, integrating enzymatic hydrolysis of cellulose and fermentation of released sugars in a single step [13]. SSF minimizes end-product inhibition, improves conversion efficiency, and reduces operational cost compared to separate hydrolysis and fermentation. The cooperative use of *A. niger* as a prolific producer of cellulases and hemicellulases, and *S. cerevisiae*, a robust ethanol-producing yeast, offers a synergistic advantage in processing complex lignocellulosic substrates such as rice husk [14].

Despite the advantages of SSF, process performance is highly sensitive to several operational variables, including pH, fermentation time, temperature, and inoculum concentration. These parameters influence enzyme activity, microbial growth, sugar release rate, and ethanol formation [15–17]. Their effects are often nonlinear and interdependent, requiring systematic optimization to achieve maximum ethanol yield. Response Surface Methodology (RSM) has been widely

recognized as a powerful statistical tool for optimizing multivariable bioprocesses, enabling the development of predictive models and reduction of experimental workload through structured design approaches [18, 19].

In this study, rice husk collected from Klaten, Indonesia, was employed as a sustainable lignocellulosic substrate for second-generation bioethanol production. SSF was conducted using *A. niger* and *S. cerevisiae*, followed by single-factor evaluation and optimization via Central Composite Design (CCD) within RSM. The aims of this study were to assess the influence of key SSF parameters, to construct a second-order predictive model describing the relationship between process variables and ethanol yield, and to identify optimal fermentation conditions for maximizing ethanol production. This study provides updated insight into rice husk valorization in Indonesia and contributes to advancing waste-to-biofuel strategies in rice-producing regions.

EXPERIMENTAL

Material

Rice husk and potato tubers used in this study were obtained from Trucuk Regency, Klaten, Indonesia. Distilled water (dH₂O) was used as the solvent in all experimental procedures. Dextrose, 0.05 M HCl, nutrient agar, yeast extract, peptone, MgSO₄·7H₂O, (NH₄)₃PO₄, and NaOH (2 M and 0.05 M) were purchased from Sigma-Aldrich, Singapore. All chemicals and reagents used were of analytical grade. The fungal strain *Aspergillus niger* and yeast *Saccharomyces cerevisiae* were obtained from the Center for Food and Nutrition Studies, Universitas Gadjah Mada (UGM), Indonesia.

Rice Husk Pre-treatment

Rice husk pretreatment was performed using an alkaline pretreatment method to enhance enzymatic accessibility. Initially, the rice husk was thoroughly washed with dH₂O to remove impurities and facilitate delignification. The rice husk was then dried in an oven until a stable weight was achieved. After dried, the rice husk was finely ground and sieved through an 80-mesh sieve to obtain a uniform particle size [20]. A total of 100 g of the ground rice husk was transferred into a 500 mL Erlenmeyer flask, followed by the addition of 300 mL of 2M NaOH solution. The mixture was agitated in an orbital shaker at 60 rpm for 30 min at ambient laboratory temperature (25-27 °C) and was subsequently left under static conditions for 1 hour to facilitate lignin removal. After the pretreatment, the sample was filtered and rinsed thoroughly with dH₂O to remove residual NaOH and lignin. The pretreated rice husk was then dried in an oven until a stable weight was obtained [21].

Simultaneous Saccharification and Fermentation (SSF) Process

Prior to optimization, the SSF process was conducted under preliminary conditions to assess baseline ethanol production. The pretreated rice husk was used as the primary substrate. The SSF was performed in a 250 mL Erlenmeyer flask containing the fermentation medium (yeast extract (10 g/L), peptone (20 g/L), and dextrose (20 g/L)), supplemented with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g/L) and $(\text{NH}_4)_3\text{PO}_4$ (0.4 g/L), which was adjusted to pH 7.0 using 0.05 M HCl or 0.05 M NaOH. The mixture was inoculated with 1.5 mL of *S. cerevisiae* and 1.5 mL of *A. niger* culture, each prepared to an optical density of $\text{OD}_{600} = 0.8$. The fermentation was carried out at a constant temperature of 30 °C in a water bath shaker set at 150 rpm to ensure adequate mixing and aeration. The incubation time was set to 96 hours, based on initial screening data. Following fermentation, the broth was filtered, and the supernatant was collected for ethanol quantification. The ethanol content was determined using gas chromatography (Shimadzu GC-2010) equipped with a flame ionization detector (FID) and an MXT-1 capillary column (100% dimethyl polysiloxane). Nitrogen was used as the carrier gas [22, 23]. Ethanol was quantified using an external calibration curve prepared from ethanol standards of known concentration. The ethanol concentration was calculated from the linear regression equation $C_{\text{EtOH}} = -1.5180 + 0.0047158A$, where C_{EtOH} is the ethanol concentration and A is the ethanol peak area obtained by GC-FID. The calibration curve showed good linearity, with $R^2 = 0.9981$. The ethanol concentration was reported as a percentage (v/v). This result served as a baseline for comparison in subsequent optimization experiments.

Single-Factor Optimization

Effect of Fermentation Time

The effect of fermentation time on bioethanol production was evaluated by varying the fermentation duration from 6 to 168 hours, with 6-hour intervals. The fermentation was conducted at a constant temperature of 35°C in a water bath shaker set at 150 rpm to ensure uniform mixing. The initial pH was adjusted to 7 before inoculation. The fermentation medium was inoculated with 1 mL of *S. cerevisiae* and 1 mL of *A. niger* as the starter culture ($\text{OD}_{600} = 0.8$)

Effect of Fermentation pH

The effect of pH on bioethanol production was evaluated by conducting fermentation at the previously optimized fermentation time and temperature. The fermentation was performed in a water bath shaker at 150 rpm, with the pH varied from 3.0 to 9.0 in increments of 0.5. The fermentation medium was inoculated with 1 mL of *S. cerevisiae* and 1 mL of *A. niger* as the starter culture ($\text{OD}_{600} = 0.8$).

Effect of Fermentation Temperature

The effect of temperature on bioethanol production was evaluated by conducting fermentation at various temperatures ranging from 25 °C to 60 °C, with increments of 5 °C. The fermentation duration was set to the optimum time determined from the preceding experiment. The fermentation was performed in a water bath shaker at 150 rpm to ensure uniform mixing and temperature stability. The pH was maintained at 7 throughout the process. A standardized inoculum of 1 mL *S. cerevisiae* and 1 mL *A. niger* was introduced into each fermentation batch ($\text{OD}_{600} = 0.8$).

Effect of Starter Concentration

The effect of starter concentration was evaluated by varying the inoculum volume of *S. cerevisiae* and *A. niger* in a ratio format (*S. cerevisiae*:*A. niger*). The inoculum volume was evaluated at 0.1 mL:0.1 mL, and followed by paired volumes ranging from 0.5 mL:0.5 mL to 4.0 mL:4.0 mL, with increments of 0.5 mL ($\text{OD}_{600} = 0.8$). The fermentation was conducted under continuous agitation at 150 rpm using a water bath shaker to maintain uniform mixing and optimal microbial interaction. The fermentation time, temperature, and pH were set to the optimal conditions determined from the previous optimization experiments.

Response Surface Methodology (RSM) Optimization

Response Surface Methodology (RSM) was employed to optimize the key variables influencing ethanol production during simultaneous saccharification and fermentation (SSF). Based on the results of single-factor experiments, four independent variables were selected for further optimization: fermentation time (X_1), pH (X_2), temperature (X_3), and starter concentration (X_4). These factors were investigated using a CCD, which enabled the evaluation of linear, quadratic, and interaction effects while reducing the overall number of experimental runs [24]. The CCD ranges were selected based on the optimum regions identified in the single-factor experiments. The design was centered at 96 h, pH 5.0, 40 °C, and paired inoculum volumes of 2.0 mL each. Symmetrical factorial intervals were established around these center points at ± 6 h for fermentation time, ± 0.5 pH unit, ± 5 °C, and ± 0.5 mL inoculum volume. For the four-factor rotatable CCD, an axial distance of $\alpha = 2$ was used, producing axial levels of 84 and 108 h, pH 4.0 and 6.0, 30 and 50 °C, and 1.0 and 3.0 mL inoculum volume.

A total of 31 experimental runs were generated by the CCD, consisting of factorial points, axial points and center points arranged in two blocks to account for experimental variability. Each run involved SSF under defined combinations of the four variables, and the ethanol content (% v/v) obtained from each experiment was used as the response variable (Y). The experimental design matrix is presented in Table 1.

The CCD data were fitted to a second-order polynomial model to describe the relationship between the process variables and ethanol yield. The general form of the quadratic regression model is expressed as:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j + \sum_{i=1}^k \beta_{ii} X_i^2 + \varepsilon$$

where Y denotes the predicted ethanol content; β_0 is the intercept; β_i , β_{ij} , and β_{ii} represent the linear, interaction, and quadratic coefficients respectively; X_i and X_j are the coded independent variables; and ε is the experimental error [25].

Model fitting and statistical analyses were conducted using R (version 4.5.0) and RStudio (version 2024.12.1 Build 563) with the “rsm” package [26–28]. Analysis of variance (ANOVA) was performed to assess the significance of model terms, overall model adequacy, and lack of fit. Regression diagnostics including coefficient of determination (R^2), adjusted R^2 , F-values, and p-values were used to evaluate the suitability of the model. In addition, response surface contour plots and three-dimensional (3D) surfaces were generated to visualise the effects of the factors and identify optimal operating regions [25].

Table 1. CCD matrix showing actual values with coded levels in parentheses and experimental and predicted ethanol concentrations.

No.	Run Order	Time (Hours)	pH	Temperature (°C)	Inoculum (mL each)	Experimental Ethanol (% v/v)	Predicted Ethanol Concentration (% v/v)
1	20	90	4.5	35	1.5	7.95	8.00
2	4	102	4.5	35	1.5	8.28	8.35
3	3	90	5.5	35	1.5	8.25	8.26
4	14	102	5.5	35	1.5	8.65	8.59
5	18	90	4.5	45	1.5	8.36	8.36
6	2	102	4.5	45	1.5	8.72	8.65
7	7	90	5.5	45	1.5	8.55	8.57
8	8	102	5.5	45	1.5	8.79	8.85
9	6	90	4.5	35	2.5	8.44	8.41
10	1	102	4.5	35	2.5	8.57	8.57
11	12	90	5.5	35	2.5	8.42	8.51
12	11	102	5.5	35	2.5	8.63	8.66
13	5	90	4.5	45	2.5	8.54	8.62
14	13	102	4.5	45	2.5	8.71	8.73
15	15	90	5.5	45	2.5	8.71	8.67
16	16	102	5.5	45	2.5	8.81	8.78
17	10	96	5.0	40	2.0	9.02	8.94
18	9	96	5.0	40	2.0	9.00	8.94
19	19	96	5.0	40	2.0	9.04	8.94
20	17	96	5.0	40	2.0	8.90	8.94
21	21	108	5.0	40	2.0	8.91	8.75
22	31	84	5.0	40	2.0	8.55	8.30
23	25	96	6.0	40	2.0	8.93	8.74
24	23	96	4.0	40	2.0	8.65	8.44
25	30	96	5.0	30	2.0	8.52	8.28
26	24	96	5.0	50	2.0	8.92	8.75
27	28	96	5.0	40	3.0	8.90	8.68
28	26	96	5.0	40	1.0	8.54	8.35
29	27	96	5.0	40	2.0	8.97	8.94
30	22	96	5.0	40	2.0	9.06	8.94
31	29	96	5.0	40	2.0	9.15	8.94

The optimal conditions predicted by the model were subsequently validated through confirmatory SSF experiments, and the model predictive accuracy was then assessed by comparing predicted and experimental ethanol yields.

Statistical Analysis

The data are presented as means $\pm$ standard deviation (SD) from a minimum of three independent experimental replicates ($n \geq 3$) unless otherwise stated. One-way analysis of variance (ANOVA) was employed to assess the significance among three or more categorical groups, and group comparisons were conducted by Student's t-test.

RESULTS AND DISCUSSION

Sample Pre-treatment

Pre-treatment of lignocellulosic biomass is a critical step in enhancing the accessibility of cellulose for enzymatic hydrolysis [29]. In this study, rice husk was subjected to a sequential physical and chemical pretreatment process to maximize its fermentable sugar potential. Initially, the raw rice husk was thoroughly washed to remove adhering soil, dust, and particulate debris. The clean biomass was then boiled and oven-dried to reduce moisture content and partially soften the structural matrix.

The dried husk was subsequently milled and sieved through an 80-mesh screen, yielding fine, uniform particles with increased surface area for enzymatic action. To achieve delignification, the ground rice husk was treated with 2 M sodium hydroxide (NaOH) under ambient conditions. This alkaline pre-treatment facilitated the solubilization of lignin, disrupted the rigid hemicellulose-lignin network, and increased cellulose exposure, which is essential for downstream saccharification [20, 21].

The application of alkaline conditions is a widely adopted strategy in lignocellulosic bioethanol processes, as it significantly enhances the efficiency of enzymatic hydrolysis by removing lignin barriers and decreasing cellulose crystallinity [30]. Rice husk also contains a substantial silica-rich inorganic fraction that may act as an additional physical barrier to the accessibility of its carbohydrate components. NaOH treatment has been reported to solubilize alkali-sensitive lignin fractions and may also remove part of the alkali-reactive silica matrix, thereby improving substrate accessibility for subsequent saccharification [31]. In this study, however, the cellulose, hemicellulose, lignin, and ash contents were not quantitatively determined before and after pretreatment. Therefore, the degree of delignification, hemicellulose solubilization, cellulose enrichment, or change in cellulose crystallinity was not directly established. The resulting pre-treated rice husk was rinsed thoroughly to neutral pH and dried to constant

weight before being used in SSF. This combination of mechanical size reduction and chemical delignification ensured optimal substrate quality for microbial fermentation and enzymatic activity in the subsequent process stages [32].

SSF Process Efficiency Before Optimization

Prior to optimization, the simultaneous saccharification and fermentation (SSF) process was carried out under preliminary conditions of 96 hours fermentation time, pH 7.0, temperature 30 °C, and an inoculum ratio of 1.5:1.5 mL (*S. cerevisiae*:*A. niger*). Each SSF experiment was conducted in a 250 mL Erlenmeyer flask containing 10.0 g of dry pretreated rice husk at a final working volume of 50 mL. Under these conditions, the process yielded an ethanol concentration of $7.43\% \pm 0.57\%$ (v/v). While this result demonstrates the fundamental viability of the SSF approach using rice husk as substrate, the suboptimal yield suggests that the selected parameters were not at their respective optima for either enzymatic hydrolysis or microbial fermentation. For instance, the chosen pH of 7.0 is above the optimal range for both cellulolytic enzyme activity and yeast fermentation, which are typically favored under mildly acidic conditions [33]. Additionally, the fermentation temperature of 30 °C may have limited the catalytic efficiency of fungal enzymes such as cellulases and amylases, which exhibit greater activity at slightly elevated temperatures (35–40 °C) [34]. Moreover, while the 1.5 mL inoculum ensured adequate microbial population, it may not have provided maximum synergistic performance between the saccharifying and fermenting organisms. These baseline conditions were intended to provide a common reference point for subsequent single-factor screening and response surface optimization. As *A. niger*-mediated substrate hydrolysis and *S. cerevisiae*-mediated ethanol fermentation may respond differently to changes in pH, temperature, fermentation time, and inoculum level, optimization was required to identify operating conditions that provided the most favorable overall ethanol-production response. The subsequent statistical optimization therefore aimed to determine an effective process-level compromise for the co-culture system rather than to establish the individual kinetic or physiological optima of the two microorganisms.

Single-Factor Optimization Results

To enhance the performance of the SSF system, a single-factor optimization approach was subsequently applied. This stepwise method enabled the evaluation of each individual variable, the fermentation time, pH, temperature, and starter concentration to determine its optimal range for maximizing ethanol production.

Effect of Fermentation Time

The effect of fermentation time on ethanol content was evaluated over a range from 6 to 168 hours.

The ethanol content increased progressively with fermentation time, peaking at 96 hours with an ethanol concentration of $8.81\% \pm 0.42\%$, followed by a plateau or slight decline thereafter (**Figure 1**). This trend suggests that the activity of *A. niger* in hydrolyzing rice husk and *S. cerevisiae* in fermenting the resulting sugars was effectively synchronized up to 96 hours. The decrease or stagnation in ethanol yield beyond the optimum point likely indicates nutrient depletion, ethanol inhibition, or reduced microbial viability in extended fermentations [35–37]. This also suggest the conversion of ethanol to acetic acid [17]. Therefore, 96 hours was identified as the optimum fermentation time under the SSF conditions employed in this study. This fermentation time is then used in the optimization process using RSM.

Effect of Fermentation pH

Fermentation pH was varied from pH 3.0 to 9.0 to assess its impact on ethanol production. The ethanol content was found to increase sharply between pH 3.0 and 5.0, reaching highest ethanol yield of $8.17\% \pm 0.32\%$ at pH 5.0 (**Figure 2**). This behavior reflects the acidophilic nature of *S. cerevisiae* and the optimal enzymatic activity of cellulases and amylases secreted by *A. niger* in mildly acidic conditions [38, 39]. Furthermore, the cellulolytic enzymes from *A. niger* also exhibit peak activity within a similar pH range [40]. At more acidic (pH < 4.0) or alkaline (pH > 6.0) conditions, reduced microbial viability and enzyme efficiency likely contributed to the lower ethanol levels. This pH is then used in the optimization process using RSM.

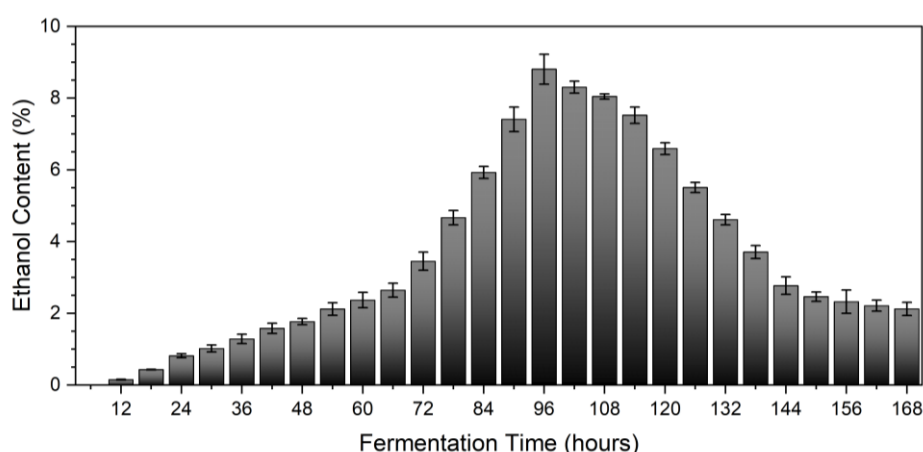


Figure 1. Effect of fermentation time on ethanol content.

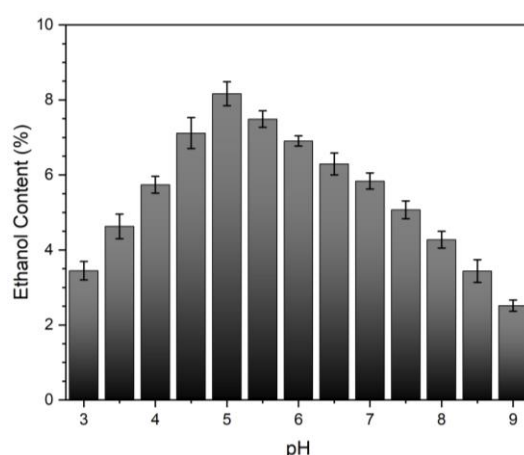


Figure 2. Effect of fermentation pH on ethanol content.

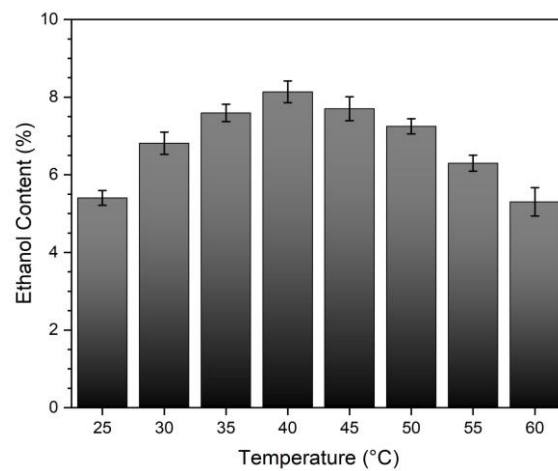


Figure 3. Effect of fermentation temperature on ethanol content.

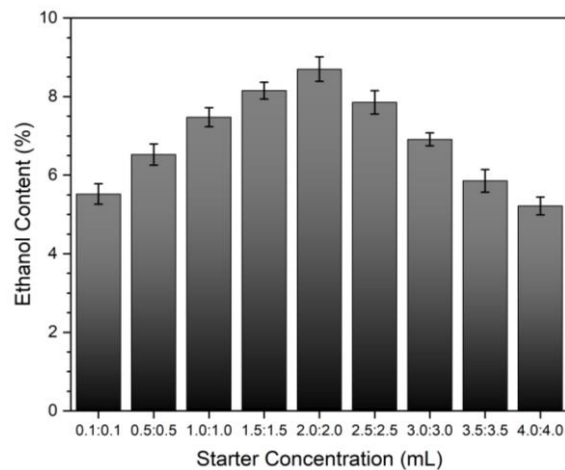


Figure 4. Effect of starter concentration on ethanol content.

Effect of Fermentation Temperature

The influence of temperature on ethanol yield was studied from 25 °C to 60 °C. Ethanol production increased steadily with temperature, with the maximum production of $8.14\% \pm 0.28\%$ achieved at 40 °C (**Figure 3**). Beyond this temperature, ethanol yield declined sharply. The observed trend reflects the balance between microbial metabolism and enzyme kinetics [41]. Higher temperatures accelerate the enzymatic hydrolysis of rice husk and enhance yeast activity, but only up to an optimum point [42]. Temperatures exceeding the optimum may lead to thermal denaturation of hydrolytic enzymes and stress-induced inhibition of yeast fermentation, therefore reducing ethanol productivity [43]. This temperature is then used in the optimization process using RSM.

Effect of Starter Concentration

The impact of varying the concentration of the microbial starter (combined *S. cerevisiae* and *A. niger*) was assessed using 0.1:0.1 mL followed by paired volumes ranging from 0.5 mL:0.5 mL to 4.0:4.0 mL (v/v). The ethanol yield increased with higher inoculum volumes, reaching a maximum of $8.70\% \pm 0.31\%$ at a concentration of 2.0:2.0 mL (**Figure 4**). Beyond this level, ethanol content declined steadily. This pattern suggests that a moderate inoculum size ensures optimal microbial interaction and substrate utilization during SSF [15]. A lower concentration may result in slower sugar consumption and incomplete fermentation, while excessive inoculum could trigger nutrient competition, oxygen limitation, or metabolic byproduct accumulation that inhibits fermentation efficiency [44, 45]. The observed decline at higher

inoculum levels (≥ 2.5 mL) may also reflect oversaturation of microbial biomass, leading to stress conditions and decreased ethanol conversion efficiency. This temperature is then used in the optimization process using RSM.

RSM Optimization and Model Validation

The preliminary RSM screening using a full factorial model and Pareto plot was employed to identify the relative importance of the four process variables prior to quadratic CCD modeling (**Figure 5**). The standardized regression coefficients showed that fermentation temperature (x_3 , $\beta = 0.125$) and fermentation time (x_1 , $\beta = 0.12125$) exerted the largest positive effects on ethanol content, whereas pH (x_2 , $\beta = 0.0775$) and starter concentration (x_4 , $\beta = 0.0800$) had smaller but still positive contributions. This pattern is reflected in the Pareto chart, where temperature and time appear as the most influential factors, indicating that ethanol production in this SSF system is particularly sensitive to these two variables.

The preliminary factorial data were initially fitted using a first-order model containing main effects and interaction terms. All interaction and higher-order terms displayed very small effects and were statistically insignificant ($p > 0.70$), suggesting that under the studied ranges the response is dominated by main effects rather than factor interactions. However, the overall factorial model (**Table 2** and **3**) exhibited a low multiple R^2 (0.4993), a strongly negative adjusted R^2 (-1.378), and a non-significant F-test ($F = 0.2659$, $p = 0.974$), indicating that a purely linear first-order interaction model could not adequately describe the system [46]. The inadequacy was attributed to substantial curvature within the investigated experimental region, which could not be represented by linear and interaction terms alone. Consequently, the factorial design was augmented with axial and additional centre points to form a CCD, and a second-order quadratic model was fitted to capture the nonlinear relationships among fermentation time, pH, temperature, starter volume, and ethanol concentration.

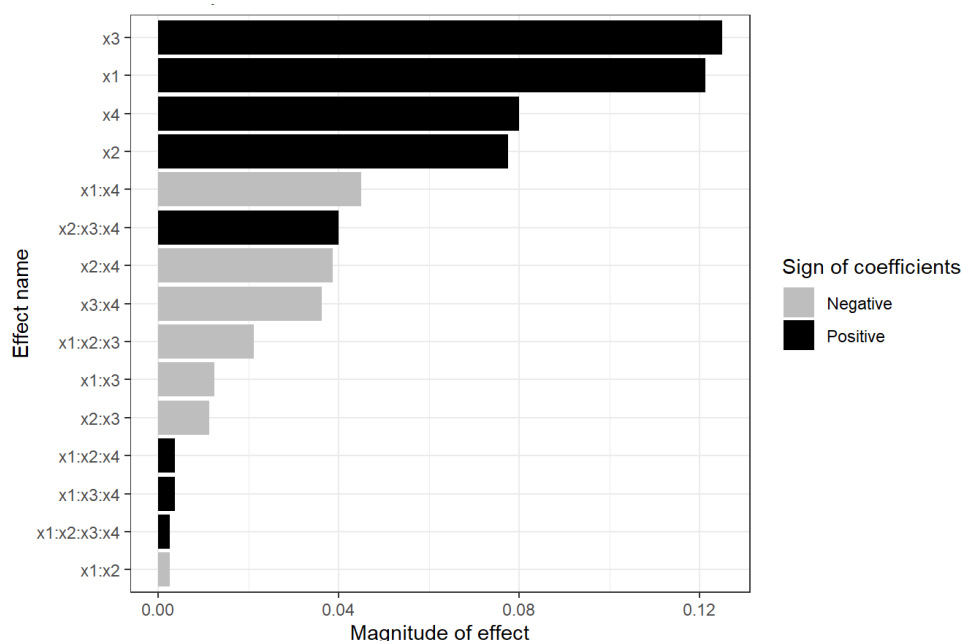


Figure 5. Pareto plot.

Table 2. Regression coefficients for the preliminary first-order model fitted to the factorial and center-point runs.

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	8.617	0.052367	164.5498	< 2e-16
x1	0.12125	0.058548	2.0709	5.60E-02
x2	0.0775	0.058548	1.3237	2.05E-01
x3	0.125	0.058548	2.135	0.04967
x4	0.08	0.058548	1.3664	1.92E-01
R-squared: 0.4539				
F-statistic: 3.117 on 4 and 15 DF, p-value: 0.04716				

Table 3. ANOVA for the preliminary first-order model fitted to the factorial and center-point runs.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
FO(x1, x2, x3, x4)	4	0.68372	0.170931	3.1165	4.72E-02
Residuals	15	0.82269	0.054846	-	-
Lack of fit	12	0.81109	0.067591	1.75E+01	0.01883
Pure error	3	0.0116	0.003867	-	-

Following the Pareto plot screening which identified fermentation time and temperature as the most influential variables, the Central Composite Design (CCD) was applied to capture nonlinear effects and refine the optimization of the SSF process [24]. The quadratic RSM model (**Table 4** and **5**) showed excellent performance, with a high R^2 value of 0.9575 and a highly significant overall F-test ($p = 1.33 \times 10^{-7}$), indicating that the model effectively explains ethanol variability across the experimental conditions. The lack-of-fit test was non-significant ($p = 0.419$), confirming that the model adequately fits the data [47, 48].

In contrast to the negligible interactions detected in the Pareto screening, most two-factor interactions in the CCD model remained statistically insignificant ($p > 0.05$), confirming that ethanol

formation in this SSF system is primarily shaped by individual factor behavior rather than strong interdependencies (**Figure 5**). The only interaction with marginal significance was time $\times$ starter concentration, suggesting a minor interplay between fermentation duration and microbial biomass. Nevertheless, the dominant pattern is the clear influence of single-factor and quadratic effects. All quadratic terms were also highly significant, demonstrating curvature in the response and indicating that each factor exhibits an optimum range beyond which ethanol production declines (**Table 5**). Most interaction terms remained statistically insignificant, supporting the earlier observation that ethanol formation in this SSF system is governed primarily by individual factor effects rather than strong synergistic interactions [49].

Table 4. Regression coefficients for the second-order quadratic model fitted to the complete CCD.

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	8.942568	0.033271	268.7797	< 2.2e-16
BlockB2	0.180676	0.030493	5.9251	2.79E-05
x1	0.110833	0.016524	6.7073	7.02E-06
x2	0.075	0.016524	4.5388	0.0003918
x3	0.116667	0.016524	7.0603	3.87E-06
x4	0.083333	0.016524	5.0431	0.0001457
x1:x2	-0.0025	0.020238	-0.1235	0.9033273
x1:x3	-0.0125	0.020238	-0.6177	0.5460686
x1:x4	-0.045	0.020238	-2.2235	0.0419639
x2:x3	-0.01125	0.020238	-0.5559	0.5864854
x2:x4	-0.03875	0.020238	-1.9147	0.0747981
x3:x4	-0.03625	0.020238	-1.7912	0.0934586
x1^2	-0.10424	0.015156	-6.8779	5.25E-06
x2^2	-0.08924	0.015156	-5.8882	2.98E-05
x3^2	-0.10674	0.015156	-7.0429	3.99E-06
x4^2	-0.10674	0.015156	-7.0429	3.99E-06
R-squared: 0.9575				
F-statistic: 22.56 on 15 and 15 DF, p-value: 1.327e-07				

Table 5. ANOVA for the second-order quadratic model fitted to the complete CCD.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Block	1	0.31378	0.313781	47.8821	4.90E-06
FO(x1, x2, x3, x4)	4	0.92315	0.230787	3.52E+01	1.84E-07
TWI(x1, x2, x3, x4)	6	0.08207	0.013679	2.09E+00	0.1161
PQ(x1, x2, x3, x4)	4	0.89812	0.224529	34.2625	2.22E-07
Residuals	15	0.0983	0.006553		
Lack of fit	10	0.0705	0.00705	1.2679	0.419
Pure error	5	0.0278	0.00556		

Based on the CCD experimental data, a second-order polynomial equation was developed to describe the quantitative relationship between the four independent variables and the ethanol yield. As indicated by the ANOVA results (**Table 5**), both linear and quadratic components made significant contributions to the model, while interaction effects were generally minor. The significance of the quadratic terms confirms the nonlinear behavior of the SSF process, consistent with the biological constraints of enzyme activity and microbial fermentation. The high R^2 value (0.9575), the significant model F-test, and the non-significant lack-of-fit collectively demonstrate that the quadratic model provides a reliable and accurate representation of ethanol production across the studied range [25, 46]. Therefore, the fitted polynomial equation can be used to predict ethanol content and to identify optimal combinations of fermentation time, pH, temperature, and starter concentration. The resulting regression model is expressed as:

$$Y = 8.942568 + 0.110833 X_1 + 0.075 X_2 + 0.116667 X_3 + 0.083333 X_4 - 0.0025 X_1 X_2 - 0.0125 X_1 X_3 - 0.045 X_1 X_4 - 0.01125 X_2 X_3 - 0.03875 X_2 X_4 - 0.03625 X_3 X_4 - 0.10424 X_1^2 - 0.08924 X_2^2 - 0.10674 X_3^2 - 0.10674 X_4^2$$

The 2D contour plots and 3D response surface graphs illustrate these relationships visually (**Figure 6** and **7**). More specifically, **Figure 7A** shows that ethanol concentration increased as fermentation time and pH approached approximately 99 h and pH 5.2, followed by a decline beyond this region. **Figure 7B** displays the most pronounced curvature, indicating the strong combined influence of fermentation time and temperature, with the maximum response located near 99 h and 42 °C. In **Figure 7C**, the tilted surface for fermentation time and paired inoculum volume reflects their negative interaction, suggesting that the beneficial effect of increasing inoculum volume diminished at longer fermentation times. **Figure 7D** shows a distinct optimum under mildly acidic conditions and temperatures in the low-40 °C range. The comparatively flatter surfaces in **Figures 7E** and **7F** indicate weaker interactions of paired inoculum volume with pH and temperature, although both surfaces retain clear maxima near 2.0-2.1 mL of each inoculum. Overall, the response surfaces indicate that ethanol concentration was governed predominantly by the quadratic effects of the four variables, with the optimum region centered at 98.80 h, pH 5.18, 42.38 °C, and 2.07 mL of each inoculum.

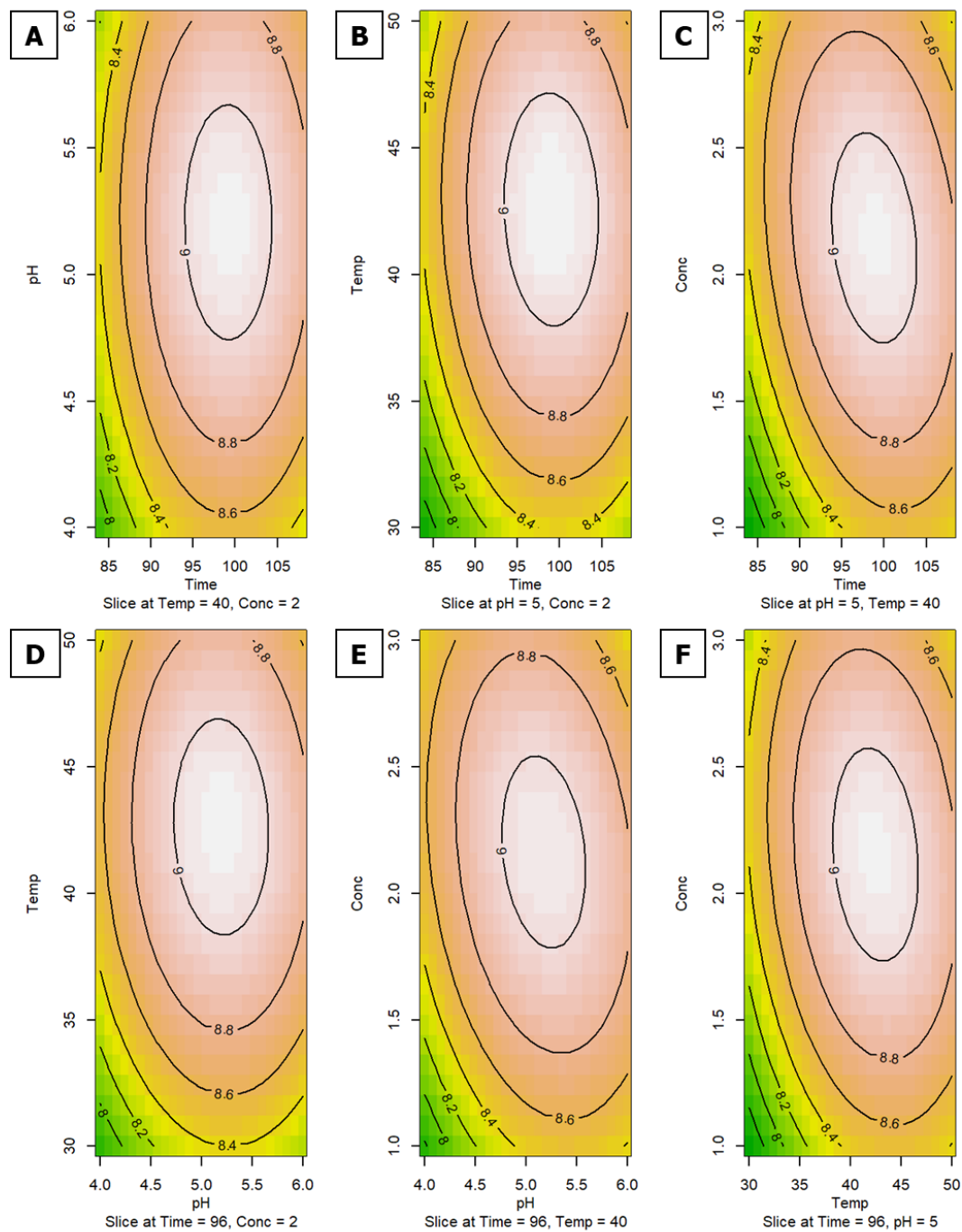


Figure 6. Two-dimensional surface plot of the RSM. (A) pH vs fermentation time, (B) temperature vs fermentation time, (C) starter concentration vs fermentation time, (D) temperature vs pH, (E) starter concentration vs pH, and (F) starter concentration vs temperature.

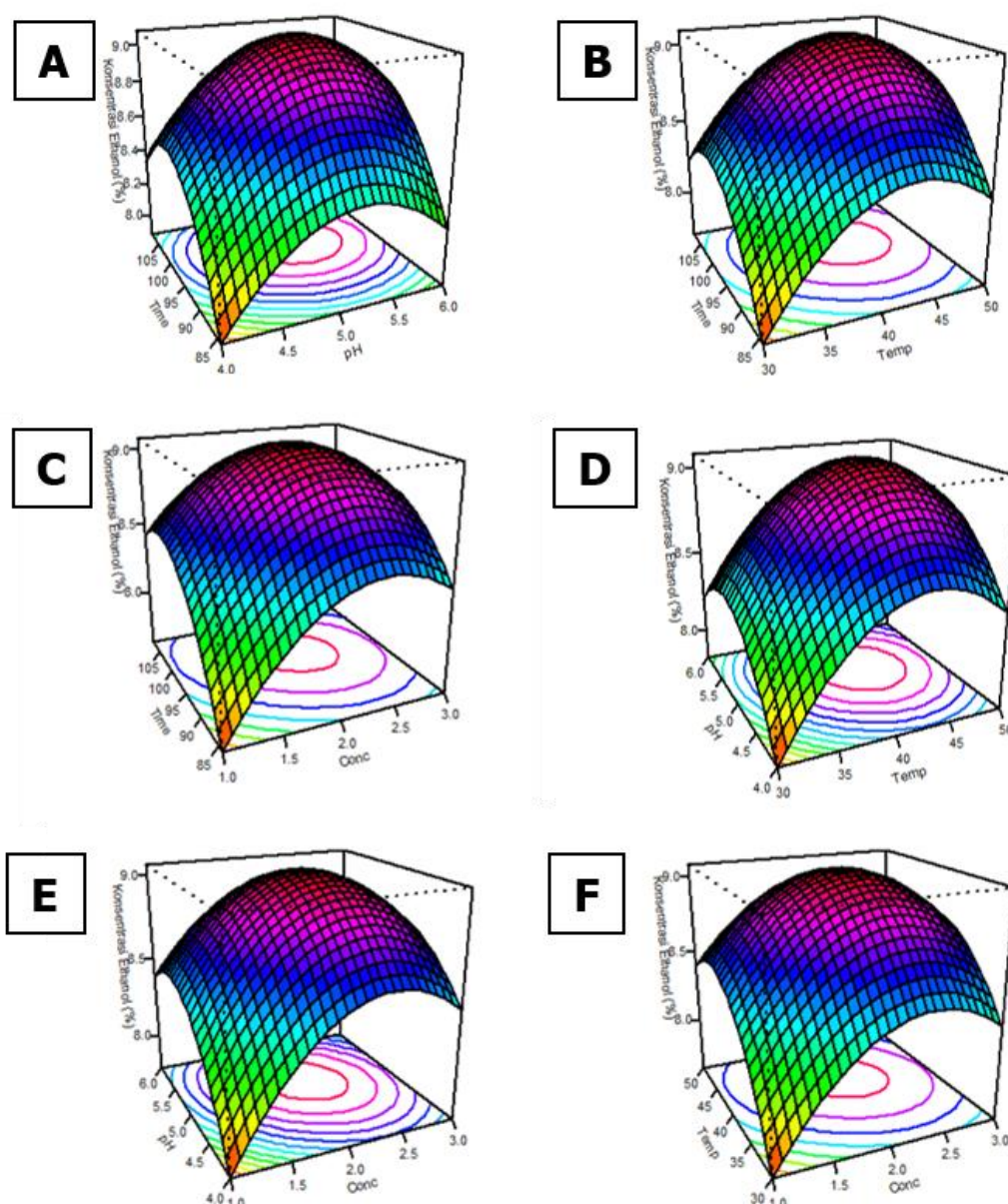


Figure 7. Three-dimensional surface plot of the RSM. (A) pH vs fermentation time, (B) temperature vs fermentation time, (C) starter concentration vs fermentation time, (D) temperature vs pH, (E) starter concentration vs pH, and (F) starter concentration vs temperature.

The model predictive accuracy was also confirmed by comparing predicted and experimental ethanol yields, which differed by only 0.00-0.25% (Table 1 and Figure 8). The run numbers on the x-axis of Figure 8 correspond to the experimental run order listed in Table 1. The highest observed ethanol concentration (9.15% (v/v)) corresponded closely with the model prediction and occurred near the central composite region (96 h, pH 5.0, 40 °C, 2.0 mL starter). The stationary point of the response surface, representing the optimum region

in the original units, was located at 98.80 h of fermentation time, with fermentation pH of 5.18, fermentation temperature at 42.38 °C and a starter concentration of 2.07 mL (*S. cerevisiae*:*A. niger*). When SSF was conducted under these optimized conditions, the ethanol yield reached $9.08 \pm 0.63\%$ (v/v), which in close agreement with the model prediction. This result represents a substantial improvement over the baseline ($7.43\% \pm 0.57\%$ (v/v)), demonstrating the effectiveness of the RSM approach and validating the conclusions drawn

from both the Pareto screening and full CCD optimization. The optimized ethanol concentration of $9.08 \pm 0.63\%$ (v/v) was equivalent to 71.64 ± 4.97 g/L, based on an ethanol density of 0.789 g/mL. The SSF system contained 10.0 g of dry pretreated rice husk in a final working volume of 50 mL, corresponding to a solid loading of 200 g/L. Accordingly, the apparent overall ethanol-to-dry-rice-husk ratio was 0.358 ± 0.025 g ethanol/g dry substrate.

Overall, the RSM results not only confirm the factor importance indicated by the Pareto plot but also reveal the nonlinear nature of the SSF system, allowing precise identification of optimal process conditions. The combined analysis shows that fermentation time and temperature are the primary determinants of ethanol productivity, while pH and starter concentration provide additional fine-tuning within their optimal ranges.

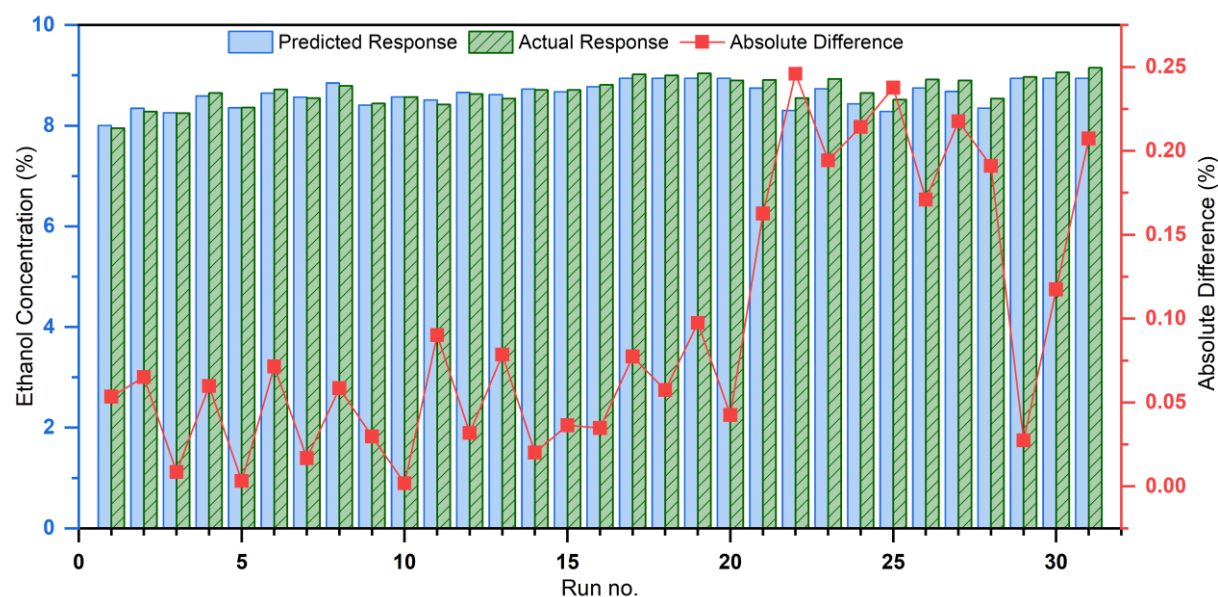


Figure 8. Actual vs predicted responses and the absolute differences from the quadratic model.

Table 6. Comparison of bioethanol production from rice-derived residues and other lignocellulosic feedstocks.

Feedstock	Pretreatment method	Microorganisms/ biocatalysts	Process type	Reported ethanol concentration	Reference
Rice-cooking wastewater	No chemical pretreatment, direct biological conversion	<i>A. niger</i> and <i>S. cerevisiae</i>	Co-culture SSF	16.97 g/L; 0.36 g/g	[50]
Sugarcane bagasse	1 N NaOH at 1.5 bar for 2 h	Cellulase, glucosidase, and <i>S. cerevisiae</i>	SSF	6.83 ± 0.07% ethanol	[51]
Sugarcane bagasse	Alkali pretreatment at high solids loading	Cellulase with <i>S. cerevisiae</i> Y-2034 or <i>Kluyveromyces marxianus</i> NCYC-587	Fed-batch SSF	75.57 ± 2.14 g/L; 66.17 ± 1.87% yield	[52]
Ciherang rice husk	2 M NaOH at ambient temperature, followed by washing and drying	<i>A. niger</i> and <i>S. cerevisiae</i>	Co-culture SSF optimized by CCD	9.08 ± 0.63% (v/v), equivalent to 71.64 ± 4.97 g/L	Present study

Table 6 compares the ethanol performance of the present system with selected SSF studies using rice-processing residues and sugarcane bagasse. The reported ethanol outputs varied substantially, reflecting differences in feedstock composition, pretreatment severity, solids loading, microbial systems, and fermentation configuration. The optimized ethanol concentration obtained in the present study, 71.64 ± 4.97 g/L, was comparable to the concentration reported for high-solids fed-batch SSF of alkali-pretreated sugarcane bagasse. However, direct comparison should be interpreted cautiously because the studies differed in feedstock characteristics, pretreatment conditions, solids loading, process configuration, medium composition, and yield basis.

CONCLUSION

This study demonstrated the potential of rice husk as a sustainable lignocellulosic feedstock for second-generation bioethanol production through simultaneous saccharification and fermentation (SSF). Single-factor analyses identified fermentation time, pH, temperature and starter concentration as critical variables influencing ethanol yield. Response Surface Methodology (RSM) using a Central Composite Design (CCD) successfully modelled and optimized the SSF process, yielding a robust quadratic model with high predictive accuracy ($R^2 = 0.9575$) and a non-significant lack-of-fit. Temperature and fermentation time were confirmed as the most influential factors, while significant quadratic terms indicated the nonlinear behavior intrinsic to enzymatic hydrolysis and microbial fermentation systems. The optimal operating conditions at 98.80 h of fermentation time, with fermentation pH of 5.18, fermentation temperature at 42.38°C and 2.07 mL (*S. cerevisiae*:*A. niger*), resulted in a maximum ethanol concentration of $9.08 \pm 0.63\%$, representing a substantial improvement over unoptimized conditions.

Overall, this work highlights the effectiveness of combining alkaline pretreatment, SSF, and statistical optimization to enhance the conversion of rice husk into bioethanol. The findings support the valorization of rice husk as a renewable energy resource and contribute to the development of waste-to-biofuel strategies relevant to Indonesia and other rice-producing regions. Future studies may focus on scaling up the process, integrating enzyme supplementation strategies, or evaluating techno-economic feasibility to further advance the practical application of this approach.

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