

The Effect of Substrate Concentration and Bacterial Loading on Biobutanol Production from Molasses Using *Clostridium acetobutylicum*

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ABSTRACT

Biobutanol is a promising alternative fuel due to its high energy content and compatibility with existing fuel infrastructure. This study evaluates the effects of molasses substrate concentration and *Clostridium acetobutylicum* loading on biobutanol production via the Acetone–Butanol–Ethanol (ABE) fermentation process. Molasses concentration was varied at 3%, 4%, 5%, 6%, and 7%, while bacterial loading ranged at 1%, 2%, 3%, 4%, and 5%. Fermentation was performed under anaerobic conditions at 37 °C for 4 days with CaCO₃ as a buffering agent. Unlike previous studies that investigated these parameters individually, this work examines their combined influence using a statistical optimization approach. The highest experimental biobutanol concentration of 6.06% was obtained at a substrate concentration of 6% and a bacterial loading of 5%. Optimization using Response Surface Methodology (RSM) produced a statistically significant quadratic model ($p < 0.0001$) with good predictive performance ($R^2 = 0.9045$; predicted $R^2 = 0.8153$). The optimal conditions were predicted at a substrate concentration of 5.220% and a bacterial loading of 4.580%, yielding a biobutanol concentration of 6.087%. These results highlight the importance of simultaneous parameter optimization for improving molasses-based biobutanol production.

INTRODUCTION

The increasing global energy demand, coupled with fossil fuel depletion and environmental concerns, has intensified the search for sustainable alternative energy sources. Long-term reliance on fossil fuels contributes to greenhouse gas emissions and climate change (Pugazhendhi et al., 2019). Consequently, renewable biofuels derived from biomass have gained attention due to their potential to reduce emissions and enhance energy security (Wang et al., 2017). Among various biofuels, biobutanol has attracted considerable interest because of its superior physicochemical properties compared to bioethanol, including higher heating value, lower vapor pressure, and better miscibility with gasoline, which improve storage safety and fuel performance (Van der Merwe et al., 2013). In addition, biobutanol is compatible with existing internal combustion engines without significant modification (Semar and Yuliarita, 2011), making it a promising candidate for next-generation transportation fuels (Ibrahim et al., 2018).

Several studies have explored biobutanol production using different substrates and fermentation conditions. Widyastuti et al. (2021) reported biobutanol production from food-grade

fructose using *Clostridium acetobutylicum*, achieving the highest concentration of 0.9058% at 15% fructose with 5 mL bacterial volume. Suprihatin et al. (2020) utilized off-grade glucose and obtained 7.785% butanol at an optimum starter volume of 11% and a fermentation time of 12 days. Meanwhile, Ni et al. (2012) employed molasses as a substrate in ABE fermentation using *Clostridium saccharobutylicum* DSM 13864 and reported a maximum butanol concentration of 13.40 g/L at 6% sugar concentration with CaCO_3 addition. However, these studies generally evaluated substrate concentration and bacterial inoculum as separate variables, without addressing their combined influence. In particular, the interaction between molasses concentration and *Clostridium acetobutylicum* loading has not been systematically investigated using a statistical optimization approach. Therefore, this study is conducted to address this gap and to determine optimal fermentation conditions for improving biobutanol production.

Biobutanol is commonly produced through Acetone–Butanol–Ethanol (ABE) fermentation using anaerobic bacteria from the genus *Clostridium*, especially *Clostridium acetobutylicum*. This process involves two metabolic phases: acidogenesis and solventogenesis (Dürre, 2008). During acidogenesis, carbohydrates are converted into organic acids, which are subsequently reassimilated into solvents—primarily butanol, acetone, and ethanol—during solventogenesis. The efficiency of this metabolic transition strongly influences final butanol yield (Xue et al., 2017), making careful control of fermentation parameters essential.

Molasses is a promising substrate for biobutanol production due to its high content of fermentable sugars, including sucrose, glucose, and fructose (Jamir et al., 2021). Its abundant availability, low cost, and presence of essential micronutrients provide economic and nutritional advantages for microbial fermentation (Ni et al., 2012). Moreover, molasses utilization supports agro-industrial waste valorization and sustainable biofuel production strategies (Huzir et al., 2018).

In addition to substrate concentration, bacterial inoculum size plays a crucial role in ABE fermentation performance. Insufficient inoculum levels can result in low metabolic activity, whereas excessive inoculum may cause rapid solvent accumulation, particularly butanol, which is toxic to *Clostridium* cells at high concentrations (Feldmane et al., 2024; Al-Shorgani et al., 2017). Maintaining a balance between substrate availability and microbial population density is therefore essential to ensure a stable transition from acidogenesis to solventogenesis (Xue et al., 2017).

To simultaneously evaluate the effects of multiple fermentation parameters and their interactions, a statistical optimization approach is required. Response Surface Methodology (RSM) enables the development of mathematical models that describe the relationship between independent variables and responses, allowing optimal conditions to be identified with fewer experimental runs (Rana et al., 2019). The effectiveness of RSM in optimizing biobutanol fermentation parameters has been well documented (Al-Shorgani et al., 2018), including its application in process simulation and scale-up studies (Haigh et al., 2018).

Based on this background, the present study aims to investigate the effects of molasses substrate concentration and *Clostridium acetobutylicum* loading on biobutanol production through ABE fermentation and to determine optimal fermentation conditions using Response Surface Methodology. The results are expected to provide insights into the interaction of key fermentation parameters and support the development of an efficient and sustainable molasses-based biobutanol production process with potential for future scale-up.

RESEARCH METHODS

This study was conducted using an experimental approach combined with statistical analysis based on Response Surface Methodology (RSM). This approach was applied to evaluate the simultaneous effects of molasses substrate concentration and *Clostridium acetobutylicum* inoculum level on biobutanol production, as well as to determine the optimal fermentation conditions in a quantitative manner (Rana et al., 2019). The main materials used in this study included molasses, *Clostridium acetobutylicum*, Reinforced Clostridial Broth (RCB), calcium carbonate (CaCO_3), and solutions of H_2SO_4 and NaOH for pH adjustment. All experiments were

carried out using standard laboratory equipment for anaerobic fermentation, including an autoclave, incubator, water bath, and filtration apparatus (Dürre, 2008).

Molasses Pretreatment

Molasses was initially diluted with distilled water at a ratio of 1:1.5 to reduce viscosity and facilitate subsequent pretreatment. The diluted molasses was then acidified using 5 M H₂SO₄ until a pH of 3.5 was reached, aiming to precipitate inorganic impurities that may inhibit fermentative microbial activity (Ni et al., 2012). The acidified solution was heated in a water bath at 60 °C for 120 minutes to enhance purification efficiency and stabilize the fermentable sugar fraction in the molasses (Jamir et al., 2021). After heating, the solution was neutralized using 5 M NaOH to pH 6.5 and filtered to remove solid residues. This pretreatment step was performed to obtain a substrate with characteristics suitable for ABE fermentation (Huzir et al., 2018).

Preparation of Bacterial Inoculum

Reinforced Clostridial Broth (RCB) medium was prepared by dissolving 3.8 g of RCB in 100 mL of distilled water, followed by sterilization at 120 °C for 15 minutes. After sterilization, *Clostridium acetobutylicum* was inoculated into the medium and incubated at 37 °C for 24 hours under anaerobic conditions. These incubation conditions were selected to promote optimal bacterial growth and metabolic activity (Al-Shorgani et al., 2017). The pH of the culture was subsequently adjusted to 6.5 to support solventogenic metabolism. Bacterial biomass was then separated by centrifugation and used as an active inoculum for the fermentation process (Xue et al., 2017).

ABE Fermentation

Pretreated molasses was diluted with distilled water to a final volume of 300 mL according to the experimental design. The molasses medium was sterilized at 120 °C for 15 minutes and cooled prior to inoculation. The fermentation medium was inoculated with the prepared bacterial culture at varying concentrations as specified in the RSM design. Calcium carbonate (CaCO₃) was added at a concentration of 0.96 g to maintain pH stability during fermentation. Fermentation was carried out at 37 °C for 4 days under anaerobic conditions, which are commonly applied for biobutanol production via ABE fermentation using *Clostridium acetobutylicum* (Van der Merwe et al., 2013).

Biobutanol Analysis and Data Processing

Biobutanol concentration was quantitatively determined using UV–Vis spectrophotometry based on a calibration curve constructed from standard butanol solutions with known concentrations. This analytical method was employed to ensure accurate and reproducible determination of butanol content in the fermentation broth (Suprihatin et al., 2020). The selection of UV–Vis spectrophotometry was based on its suitability for rapid, cost-effective, and reproducible quantification when combined with proper calibration and method validation. Since the primary objective of this study was to evaluate the relative effects of substrate concentration and bacterial loading and to optimize biobutanol production using Response Surface Methodology (RSM), this work is justified for process optimization purposes rather than detailed solvent composition analysis, so relative alcohol quantification was considered sufficient. The analytical reliability method was supported by the use of calibration standards with known biobutanol concentrations and consistent measurement procedures. Method accuracy and precision were addressed through the agreement and repeatability of absorbance values obtained from calibration and sample measurements. Method sensitivity, corresponding to the limit of detection, was sufficient for the concentration range investigated, as all measured biobutanol concentrations were above the instrumental noise level and within the linear range of the calibration curve. The experimental data were subsequently analyzed using Response Surface Methodology to develop a mathematical model describing the relationship between process variables and biobutanol production. The model was used to identify optimal fermentation conditions and to evaluate the statistical significance of individual variables and their interactions (Al-Shorgani et al., 2018).

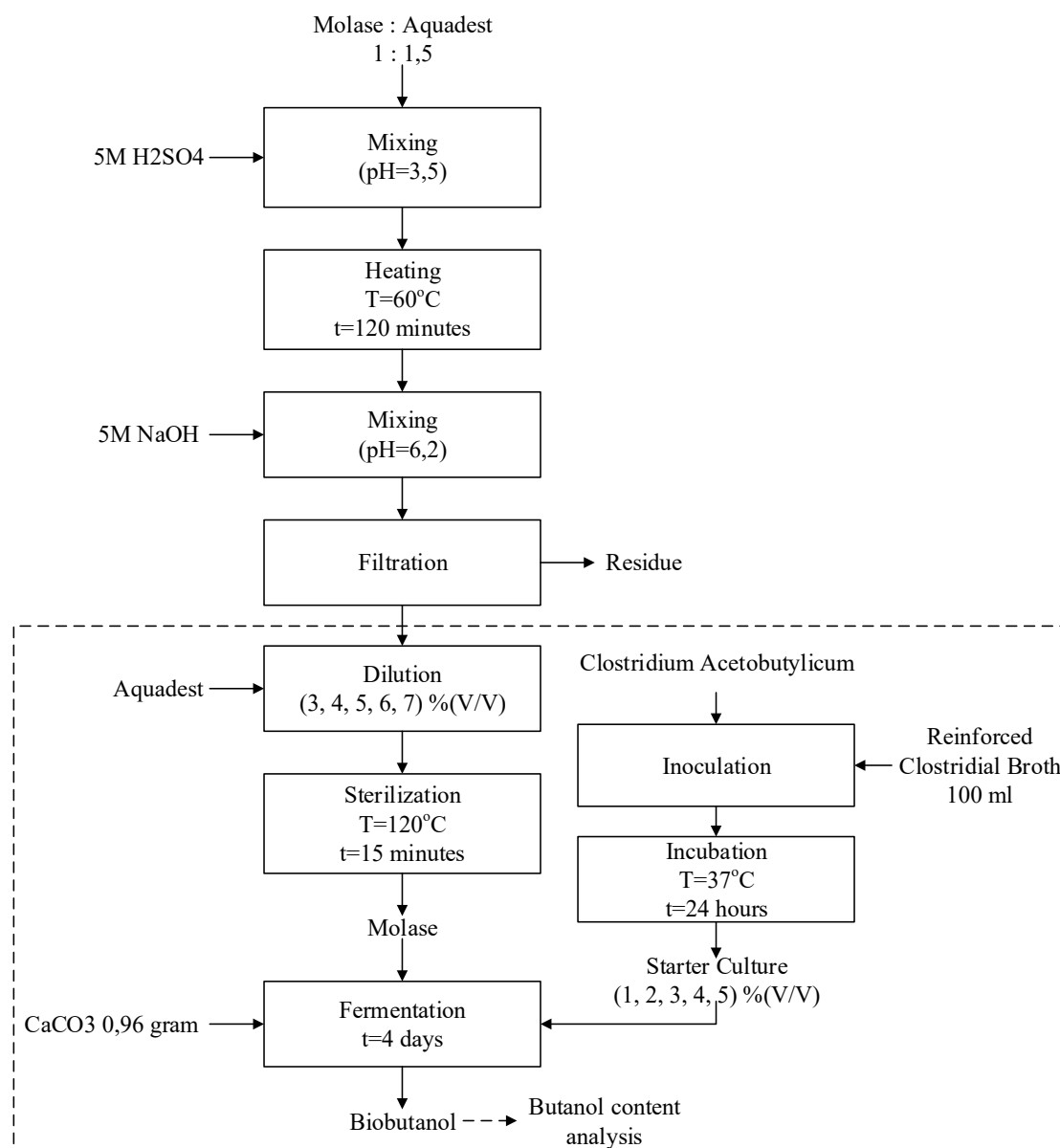


Figure 1. Research Process Flowchart

Experimental Design and Statistical Analysis

The experimental design was developed using a face-centered Central Composite Design (CCD) with $\alpha = 1$, in which all factors were evaluated at coded levels of -1 , 0 , and $+1$ to evaluate the effects of substrate concentration (X_1) and bacterial loading (X_2) on biobutanol production. A total of 14 experimental runs were conducted, including 3 center points to ensure data reliability. Statistical analysis was carried out using Design Expert. The developed model was evaluated using analysis of variance (ANOVA). The significance of the model and its individual terms was determined based on p-values at a 95% confidence level. The goodness of fit of the model was assessed using the coefficient of determination (R^2), adjusted R^2 , and lack-of-fit analysis.

RESULTS AND DISCUSSION

Effect of Substrate Concentration and Bacterial Amount on Biobutanol Production

Biobutanol concentration was determined using UV-Vis spectrophotometry at a wavelength of 250 nm. The research results indicate that variations in molasses substrate concentration and the amount of *Clostridium acetobutylicum* exert a significant influence on the biobutanol concentration produced. In the Acetone-Butanol-Ethanol (ABE) fermentation system,

substrate availability determines the carbon supply, whereas bacterial loading affects the intensity of metabolic activity leading to solvent formation (Dürre, 2008). The interaction between these two parameters plays a critical role in regulating the balance between the acidogenic and solventogenic phases throughout fermentation. Alterations in this balance directly affect the measurable biobutanol concentration. The biobutanol concentrations obtained under different combinations of substrate concentration and bacterial amount are presented in Table 1 as the basis for further analysis.

Tabel 1. Results of Butanol Concentration Analysis at Various Substrate and Bacterial Concentrations

Substrate Concentration (%)	Bacterial Loading (%)	Butanol Content (%)
3	1	3.55
	3	4.38
	5	5.00
5	1	4.39
	3	5.40
	3	5.61
	3	5.61
	5	5.98
6	1	4.90
	3	5.72
	5	6.06
7	1	3.45
	3	4.64
	5	5.40

Based on the data in Table 1, increasing the molasses substrate concentration generally resulted in higher biobutanol concentrations up to a certain condition. The highest biobutanol concentration, 6.06%, was obtained at a substrate concentration of 6% with a bacterial loading of 5%. This condition indicates that an adequate supply of fermentable sugars supports organic acid formation during the initial stage of fermentation and promotes their conversion into solvents in the subsequent stage. Molasses contains sucrose, glucose, and fructose, which are readily metabolized and capable of sustaining carbon flux throughout the fermentation process (Ni et al., 2012). In addition, a sufficient bacterial population ensures adequate enzymatic capacity to support efficient butanol formation (Jamir et al., 2021).

However, increasing substrate concentration beyond the optimal range does not necessarily lead to a proportional increase in biobutanol concentration. At excessively high substrate levels, the fermentation system tends to experience metabolic imbalance, which reduces solvent formation efficiency. Such conditions may induce increased osmotic pressure and accumulation of intermediate metabolites that interfere with cellular physiological activity. These effects can inhibit the solventogenic phase and ultimately reduce the final butanol yield (Al-Shorgani et al., 2015). Therefore, controlling substrate concentration is a crucial aspect of maintaining optimal fermentation performance.

Interaction between Substrate Concentration and Bacterial Amount

The relationship between substrate concentration and bacterial amount with respect to biobutanol concentration is illustrated in Figure 2. At low substrate concentrations, the biobutanol concentration remained relatively low even when the bacterial amount was increased. This behavior indicates that limited carbon supply constitutes the primary limiting factor in solvent formation. Insufficient substrate restricts acid formation during the early fermentation stage, thereby reducing the availability of precursors required for solventogenesis (Xue et al., 2017). Consequently, increasing bacterial loading does not significantly enhance biobutanol concentration under these conditions.

As substrate concentration increases, a more pronounced increase in biobutanol concentration is observed. This trend reflects enhanced metabolic activity and improved efficiency in the conversion of acids into solvents within the fermentation system. Nevertheless, at higher

substrate concentrations, the increase in biobutanol concentration gradually slows and approaches a plateau. This pattern indicates that the fermentation system is nearing a saturation condition. In addition, the accumulation of butanol at elevated concentrations can reduce cell viability due to the toxic effect of butanol on *Clostridium* cell membranes (Al-Shorgani et al., 2017).

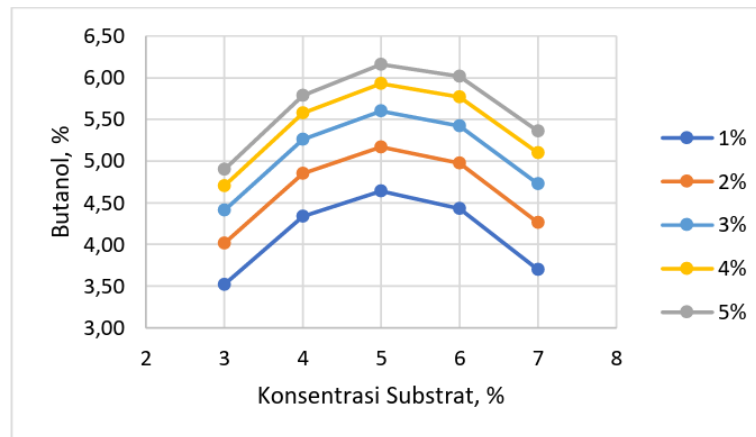


Figure 2. Effect of substrate concentration and bacterial amount on biobutanol concentration

Based on Figure 2, at low substrate concentrations, the biobutanol concentration produced was relatively low. This behavior can be attributed to limited nutrient availability, which restricts bacterial growth and metabolic activity, resulting in reduced solvent formation (Dürre, 2008). As the substrate concentration increased, biobutanol production improved significantly, indicating enhanced metabolic activity and more effective solventogenesis.

However, at higher substrate concentrations, the increase in biobutanol concentration tended to level off, suggesting that the fermentation system approached a saturation condition. In addition, high solvent concentrations, particularly butanol, are known to be toxic to *Clostridium* species, as they disrupt cell membrane integrity and inhibit essential metabolic processes (Al-Shorgani *et al.*, 2017). This inhibitory effect likely contributed to the observed plateau in biobutanol production at elevated substrate levels.

Statistical Analysis of RSM Model

Table 2. Analysis of Variance (ANOVA) for the Quadratic RSM Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8.53	5	1.71	25.64	< 0.0001	significant
A-Konsentrasi Subtrat	0.1692	1	0.1692	2.54	0.1496	
B-Konsentrasi Bakteri	4.49	1	4.49	67.45	< 0.0001	
AB	0.0221	1	0.0221	0.3326	0.5800	
A ²	2.96	1	2.96	44.42	0.0002	
B ²	0.1300	1	0.1300	1.95	0.1998	
Residual	0.5327	8	0.0666			
Lack of Fit	0.5033	6	0.0839	5.71	0.1566	not significant
Pure Error	0.0294	2	0.0147			
Cor Total	9.07	13				

Analysis of variance (ANOVA) was performed to evaluate the significance of the developed quadratic RSM model. The results showed that the model was statistically significant ($p < 0.0001$), indicating that substrate concentration and bacterial loading significantly affected

biobutanol production. The interaction between both variables also showed a significant effect on the response. The quadratic model also showed a good fit with an R^2 predicted value of 0.8153 and an adjusted R^2 of 0.9045, indicating that the model could adequately explain the variability of biobutanol production.

Based on the regression analysis, the relationship between substrate concentration (X_1) and bacterial loading (X_2) on biobutanol production (Y) can be described by the following second-order polynomial equation:

$$Y = -2.55794 + 2.60068X_1 + 0.589654X_2 + 0.017786X_1X_2 - 0.257358X_1^2 - 0.049776X_2^2 \quad (1)$$

The regression model indicates that both have positive linear effects on biobutanol production, as reflected by their positive coefficients. The negative quadratic terms suggest the presence of an optimum region, where further increases in the variables may reduce biobutanol produced due to substrate inhibition.

Optimization of Biobutanol Production Using Response Surface Methodology (RSM)

To determine the optimal fermentation operating conditions, the experimental data were analyzed using Response Surface Methodology (RSM). This approach allows simultaneous evaluation of the effects of molasses substrate concentration and *Clostridium acetobutylicum* loading, as well as their interactions, on biobutanol production. In ABE fermentation, the relationship between process variables and the response is generally nonlinear, making conventional statistical methods insufficient to describe system behavior (Myers et al., 2016). RSM addresses this limitation by employing second-order polynomial models capable of accurately representing response changes under varying operating conditions, and it has been widely applied in bioprocess optimization involving complex biological interactions (Rana et al., 2019).

Furthermore, RSM facilitates the identification of an optimal operating region that balances substrate availability and microbial metabolic activity. Substrate concentration influences carbon flux, while bacterial loading determines enzymatic capacity during solvent formation; imbalance between these parameters may lead to substrate limitation or cellular inhibition due to solvent toxicity (Xue et al., 2017). By systematically analyzing variable interactions, RSM enables rational determination of conditions that maximize biobutanol yield. This approach has been extensively applied in solvent fermentation optimization and has proven effective for process evaluation and scale-up development (Al-Shorgani et al., 2018; Haigh et al., 2018).

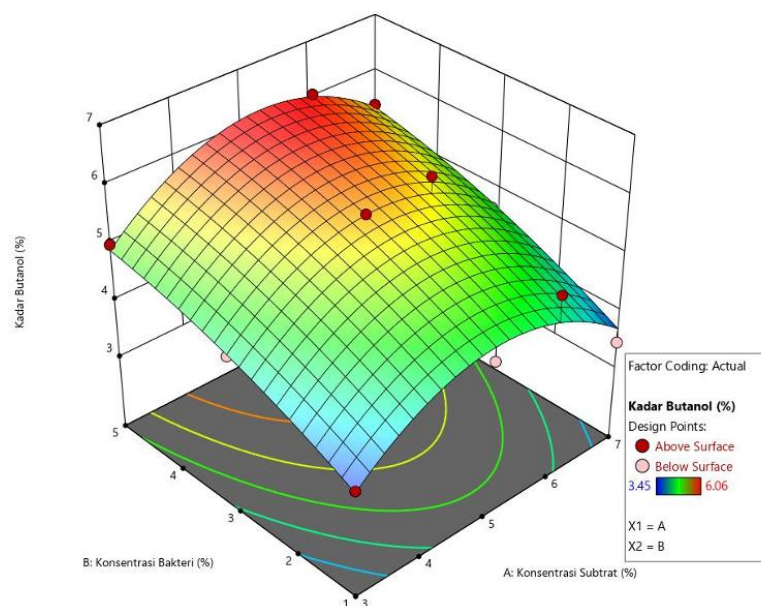


Figure 3. 3D surface plot of substrate concentration versus bacterial concentration on butanol content

The three-dimensional (3D) response surface plot shown in Figure 3 illustrates the combined effects of substrate concentration and bacterial amount on biobutanol production. This visualization provides a clearer understanding of variable interactions than single-factor analysis and is commonly used to identify optimal operating regions in ABE fermentation systems (Rana et al., 2019). Variations in substrate concentration and inoculum level jointly influence microbial metabolism by regulating the balance between acidogenesis and solventogenesis.

As shown in Figure 3, the highest biobutanol concentration (6.06%) was achieved at a substrate concentration of 6% and a bacterial amount of 5%, indicating a balanced relationship between carbon availability and microbial metabolic capacity. At this condition, sufficient fermentable sugars supported acid formation, which was subsequently converted into solvents during the solventogenic phase. Similar trends in molasses-based fermentation systems have been reported previously (Ni et al., 2012).

The relatively high bacterial amount under optimal conditions enhanced enzymatic availability, facilitating efficient conversion of organic acids into solvents and maintaining metabolic stability during ABE fermentation. The importance of adequate *Clostridium acetobutylicum* population in improving solvent production has also been observed in fermentation studies using other sugar-based substrates (Van der Merwe et al., 2013).

In contrast, the lowest biobutanol concentration 3.45% occurred at a substrate concentration of 7% with a bacterial amount of 1%. This result indicates that increasing substrate concentration alone is insufficient to improve biobutanol production without adequate inoculum levels. Excess substrate may increase osmotic stress, while limited bacterial populations reduce solvent conversion capacity, leading to decreased fermentation efficiency (Al-Shorgani et al., 2015). Additionally, solvent accumulation at higher concentrations may inhibit microbial activity due to butanol toxicity (Al-Shorgani et al., 2017).

Table 3. Optimization Results of Butanol Concentration Response

Number	Substrate Concentration	Bacterial Loading	Butanol Content	Desirability	
1	5.220	4.580	6.087	1.000	Selected

The RSM analysis generated a second-order polynomial model that describes the Response Surface Methodology analysis generated a second-order polynomial model describing the relationship between substrate concentration, bacterial amount, and biobutanol concentration. This model was used to predict the optimal operating conditions within the investigated variable ranges. Based on the optimization results, the optimum condition was predicted at a substrate concentration of 5.220% and a bacterial amount of 4.580%. Under these conditions, the predicted biobutanol concentration reached 6.087%, with a desirability value of 1.000. A high desirability value indicates that the defined optimization criteria were fully satisfied (Al-Shorgani et al., 2018).

The agreement between the predicted values and the maximum biobutanol concentration obtained from the research data indicates that the RSM approach adequately represents the behavior of the fermentation system. This method allows quantitative evaluation of process variables and their interactions without requiring an excessive number of experimental runs. The application of RSM in biobutanol fermentation studies is widely recognized for its effectiveness in identifying optimal process conditions. Moreover, this approach provides a solid foundation for further process development at larger scales. The reliability of RSM as a bioprocess optimization tool has been well established in previous studies (Myers et al., 2016).

Presentation of Formulas

The biobutanol content was calculated based on the analysis results of the fermentation products using the following equation:

$$\text{Biobutanol Content (\%)} = \frac{\text{Amount of biobutanol produced}}{\text{Total volume of fermentation broth}} \times 100\% \quad (2)$$

The relationship between the independent variables and the response in RSM optimization is expressed in the form of a second-order polynomial equation as follows::

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (3)$$

where Y represents biobutanol content (%), X_1 is substrate concentration (%), and X_2 is bacterial loading (%).

CONCLUSION

This study confirms that both molasses substrate concentration and the amount of *Clostridium acetobutylicum* significantly influence biobutanol production through ABE fermentation. An appropriate substrate concentration provides sufficient fermentable sugars to support bacterial metabolic activity, while an adequate inoculum level ensures that the fermentation process proceeds effectively. Experimental results showed that the highest biobutanol concentration was obtained at a substrate concentration of 6% and a bacterial level of 5%, reaching 6.06%. This condition indicates that a proper balance between substrate availability and bacterial population effectively promotes the solventogenic phase, leading to enhanced butanol formation. However, increasing the substrate concentration beyond the optimal range did not result in a substantial improvement in biobutanol yield, suggesting limitations in the bacterial capacity to convert excess substrate during fermentation.

Optimization using Response Surface Methodology (RSM) identified optimal conditions at a substrate concentration of 5.220% and a bacterial level of 4.580%, with a predicted biobutanol concentration of 6.087%. The quadratic model showed a good fit with an R^2 predicted value of 0.8153 and an adjusted R^2 of 0.9045, indicating that the model could adequately explain the variability of biobutanol production. This predicted value is very close to the experimental maximum, demonstrating that the RSM model accurately represents the fermentation system and is reliable for determining optimal operating conditions. Based on these findings, molasses can be considered an effective and economical feedstock for biobutanol production. Furthermore, the application of RSM proved to be a valuable tool for optimizing fermentation parameters and has strong potential to improve the efficiency of biobutanol production in future scale-up applications.

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