# Optimization of Biobutanol Production from Poplar Wood Hydrolysate using a Mutant of *Clostridium saccharobutylicum*

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Enzymatic hydrolysate of poplar wood was used in this study to produce biobutanol by a mutant M-18. Plackett-Burman and Box-Behnken designs were adopted in order to screen crucial factors from various nutrient factors affecting butanol production. These factors included reducing sugar content of hydrolysate, $MgSO_4 \cdot 7H_2O$, yeast extract, $K_2HPO_4$, $FeSO_4 \cdot 7H_2O$, $CaCO_3$, and ammonium sulfate. The results demonstrated that a reduction in sugar content, $K_2HPO_4$, and $CaCO_3$ were the most critical factors. Yeast extract was also found to have a significant effect on biobutanol production by performing an analysis of variance (ANOVA). Optimal variables were 44.53 g/L of reducing sugar concentration, 1.36 g/L of $K_2HPO_4$, and 4.65 g/L of $CaCO_3$ according to the Box-Behnken design. A model was established and used to predict a maximum biobutanol production of 7.59 g/L. Optimal conditions of fermentation were determined by orthogonal tests. Three distinct factors with important effects on biobutanol production were explored. The pH was identified as having the most significant effect on biobutanol biosynthesis. Optimized fermentation conditions for biobutanol production were determined at an initial pH of 6.5, temperature 36 °C, and inoculum quantity 9%. Under these conditions, a maximum biobutanol production of 8.41±0.20 g/L was achieved in verification experiments in a 3 L fermentation tank.

*Keywords: Plackett-Burman design; Box-Behnken designs; Medium optimization; Orthogonal tests; Fermentation conditions optimization*

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## INTRODUCTION

Utilization of renewable biomass, such as agricultural residue and lignocellulosic wastes from the forestry industry, to produce biofuels has generated new interest due to the growing energy demands coupled with the limited petroleum resources and other environmental concerns (Du *et al*. 2013; Li *et al*. 2014). Biobutanol produced from renewable feedstock through microbial fermentation is considered to be a promising energy source and has potential to be a substitute for gasoline (Al-Shorgani *et al*. 2013).

Biobutanol is more sustainable and has superior characteristics compared to bioethanol. It contains a high energy density, has less volatility and hygroscopicity, is less corrosive, and it can be used in current combustion engines without any modifications.

Also, biobutanol can be fully dissolved in gasoline with arbitrary proportion (Jang *et al*. 2012; Gottumukkala *et al*. 2013). Biobutanol can be produced through the acetone-butanol-ethanol (ABE) fermentation using solventogenic *Clostridium* strains. However, ABE fermentation faces several challenges at present. One of them is the high cost of substrates, which is an important part for the overall cost of biobutanol production (Ranjan and Moholkar 2012).

Abundant and inexpensive feedstocks are desirable for biobutanol production. As one of the main plantation-planted tree species in China, the planting scale of poplar is the largest in the world (Liang *et al*. 2006). So, poplar wood is considered an abundant and readily available lignocellulosic material for the production of biobutanol. The fermentable sugars obtained from poplar wood hydrolysate are mainly composed of glucose, xylose, and arabinose, which are all easily degraded during the processes of ABE fermentation. On the other hand, ABE fermentation is a biphasic process, the complexity of which is influenced by a number of factors. Wang and Blaschek (2011) found that the cells are very sensitive to certain factors. The pH is a critical factor that influences the yield of biobutanol. Proper pH can urge the metabolic shift from acidogenesis to solventogenesis (Liao *et al*. 2015). In addition, sugar concentration in the fermentation substrate has an important influence on biobutanol yield. A low sugar concentration can reduce cell growth, interrupt the acidogenesis phase, and further affect solvent formation; a high sugar concentration can directly inhibit cell growth and cause failure of the whole ABE fermentation (Ezeji *et al*. 2005). Additionally, a sufficient amount of nitrogen and a proper carbon to nitrogen ratio are essential for generating new cells to ensure the smooth progression of ABE fermentation (Madihah *et al*. 2001; Ibrahim *et al*. 2012). Thus, in order to increase the production of solvents, an optimization study is very important, especially in obtaining a high biobutanol yield from cheap biomass as well as balancing any parameters that may inhibit the cell's metabolism and solvents formation.

The utilization of sugars from poplar wood hydrolysate was successfully demonstrated in the course of a previous study by the authors (Wang *et al*. 2015) for biobutanol production by *Clostridium saccharobutylicum* ATCC BAA-117. The main objective of the present work was to further investigate the optimum conditions and to increase the biobutanol yield by a mutant *Clostridium saccharobutylicum* ATCC BAA-117. Plackett-Burman design (PBD), as a statistical approach, has been used for identifying significant nutritional variables and screening the effect of nutrient factors influencing biobutanol production. The positive significant variables were further optimized using response surface methodology (RSM), which is a statistical approach to evaluate the relative significance between independent variables, to reveal the interactions of the variables and then determine the optimal conditions (Bezerra *et al*. 2008). Simultaneously, the fermentation conditions were optimized by orthogonal tests. These approaches have the advantage of reducing the number of experiments required. All of these approaches have been shown to work effectively in the bioprocess industry (Vishwanatha *et al*. 2010).

## MATERIALS AND METHODS

### Microorganism

The organism used in this study was a mutant strain M-18, which was acquired through ultraviolet mutagenesis by *Clostridium saccharobutylicum* ATCC BAA-117. The

specific treatment method was using the 20 W UV lamp to process the bacteria suspension for 100 seconds at a distance of 20 cm. Then the specimens were evaluated through the bromocresol purple (BCP) medium screening, fermentation re-screening, determination of the genetic stability of subculture.

## Media Preparation and Fermentation Experiments

The growth medium used for the pre-cultures contained 10 g/L of beef extract, 10 g/L of tryptone, 3 g/L of yeast extract, 5 g/L of glucose, 3 g/L of $C_2H_9NaO_5$•$3H_2O$, 5 g/L of NaCl, 0.5 g/L of L-cysteine hydrochloride, and 1 g/L of soluble starch. The modified TYA medium used as a seed medium contained 25 g/L of glucose, 3.0 g/L of tryptone, 1.5 g/L of yeast extract, 3.0 g/L of $CH_3COONH_4$, 0.37 g/L of $MgSO_4$•$7H_2O$, 1.0 g/L of $K_2HPO_4$, 0.015 g/L of $FeSO_4$•$7H_2O$, and 2 g/L of $CaCO_3$. The fermentation media consisted of the following components: the poplar hydrolysates (50 g/L reducing sugar, which contained glucose 27.5g, xylose 19.5g and arabinose 3 g) (Wang *et al*. 2015), 1.5 g/L of yeast extract, 3.0 g/L of $CH_3COONH_4$, 0.37 g/L of $MgSO_4$•$7H_2O$, 1.0 g/L of $K_2HPO_4$, 0.015 g/L of $FeSO_4$•$7H_2O$, and 4 g/L of $CaCO_3$. The prepared media were autoclaved at 115 °C for 20 min. The fermentation process was carried out according to Wang *et al*. (2015). 250 mL screw-capped bottles containing 150 mL of the fermentation medium and 3 L fermentation tanks containing 2 L fermentation broth were used. The inoculation quantity was 10% in all experiments. The pH was adjusted using 1-M KOH or 1-M HCl according to the experimental need. The whole fermentation process was incubated at 37 °C in an anaerobic chamber or an anaerobic fermentator of sludge for 96 h. A 1 mL of sample was collected for analysis.

## ABE and Acids Analysis

The ABE concentrations were measured using gas chromatography (GC-2010, SHIMADZU, Japan) equipped with a flame ionization detector (FID) and an Agilent HP-INNOWAX capillary column (30 mm × 0.25 mm × 0.25 μm). The column temperature was maintained at 60 °C. The injector and detector temperatures were maintained at 180 and 210 °C, respectively. Isobutanol was used as the internal standard, and nitrogen gas was the carrier. The acids (acetic and butyric acids) were determined by HPLC (HPLC-1210, Japan) with an Aminex HPX-87H column. 0.2-M $H_3PO_4$ was used as the mobile phase at a flow rate of 1 mL/min, and the temperature was maintained at 30 °C. All samples were centrifuged at 10000 rpm for 10 min and filtered before being analyzed.

## Plackett-Burman Design (PBD)

The most significant nutritional factors affecting biobutanol production by a mutant strain M-18 from *Clostridium saccharobutylicum* BAA-117 were screened. PBD was used, in which each independent variable was investigated at a high and low level, indicated by +1 and -1, respectively. The design details are shown in Table 1. The *P* value of less than 5% was considered to be a significant parameter affecting biobutanol production. The PBD experimental design was created using Minitab17 software (Minitab Inc., USA). The design involved 7 factors, namely: reducing sugar content, $MgSO_4$•$7H_2O$, yeast extract, $K_2HPO_4$, $FeSO_4$•$7H_2O$, $CaCO_3$, and ammonium sulfate. The aforementioned factors were coded as $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$, and $X_7$, respectively.

**Table 1.** The Level of Variables in the Plackett-Burman Design Affecting Biobutanol Production

| Code | Factor | Low level (-1) | High level (+1) |
|---|---|---|---|
| $X_1$ | Reducing sugar | 40 | 50 |
| $X_2$ | $MgSO_4•7H_2O$ | 0.37 | 0.46 |
| $X_3$ | Yeast extract | 1.5 | 1.88 |
| $X_4$ | $K_2HPO_4$ | 1 | 1.25 |
| $X_5$ | $FeSO_4•7H_2O$ | 0.015 | 0.019 |
| $X_6$ | $CaCO_3$ | 4.45 | 5.56 |
| $X_7$ | Ammonium sulfate | 3 | 3.75 |

In this PBD, 12 runs were done, as illustrated in Table 3.

Calculations of the effect of individual factors on biobutanol production were based on the following equation,

$$Y = \beta_0 + \Sigma\beta_i X_i \quad (1)$$

where $Y$ is biobutanol production under the effect of the study factors, $X_i$ represents the coded independent variables, and $\beta_0$ and $\beta_i$ are the constant coefficients. The significance of the fitted model and the significance of the effect of individual factors on biobutanol production were evaluated by an analysis of variance (ANOVA).

## Box-Behnken Design

To further study the most significant variables screened in the PBD experiment affecting biobutanol production, a Box-Behnken design was applied, which can reflect the nature of the response surface in the experimental region and identify the optimum conditions for biobutanol production (Du *et al*. 2013). The design matrix was established by Design-Expert version 8.0.5b software (State-Ease Inc., USA), consisting of 17 trials presented in Table 2. Each variable was studied on three levels: low, middle, and high values, coded -1, 0, and +1, respectively. The levels of each variable were selected based on the Plackett-Burman design. The relationship between the independent variables and the response was fitted to a second-order polynomial function, and the optimal point was predicted. For three factors, the equation was,

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2 \quad (2)$$

where $Y$ is the predicted response, $\beta_0$ is the constant coefficient; $\beta_1$, $\beta_2$, and $\beta_3$ are the linear coefficients; $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ are the interaction coefficients; $\beta_{11}$, $\beta_{22}$, and $\beta_{33}$ are the quadratic coefficients; and $X_1$, $X_2$,and $X_3$ are the coded variables.

**Table 2.** The Levels of Variables in Box-Behnken Design

| Factor | Level | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| $X_1$：$K_2HPO_4$ (g/L) | 0.5 | 1 | 1.5 |
| $X_2$：Reducing sugar content (g/L) | 30 | 40 | 50 |
| $X_3$：$CaCO_3$(g/L) | 3 | 4 | 5 |

## Orthogonal Optimization and Verification

A $L_9$ ($3^4$) orthogonal array design with three factors at three levels consisting of 9 different experimental trials was used for optimization of biobutanol fermentation by M-

18. Three factors were at initial pH, temperature, and inoculum size. Their assigned levels and the experimental design along with biobutanol production data are listed in Table 8. Statistical analysis of variance (ANOVA) with SPSS Statistics 19.0 (IBM software, USA) was carried out to see whether these parameters were statistically significant. The results are shown in Tables 9 and 10.

## RESULTS AND DISCUSSION

### Screening of the Important Parameters Affecting Biobutanol Production

The effects of the seven medium nutrients, namely, the reducing sugar content of poplar wood hydrolysate, $MgSO_4 \cdot 7H_2O$, yeast extract, $K_2HPO_4$, $FeSO_4 \cdot 7H_2O$, $CaCO_3$, and ammonium sulfate on biobutanol production in batch culture of newly mutant *Clostridium saccharobutylicum* (M-18) were tested by PBD. The experimental and predicted values of these nutrient components in the production of biobutanol are reflected in Table 3.

Statistical analysis showed that the effects of reducing sugar concentration ($P = 0.000$), $K_2HPO_4$ ($P = 0.000$), $CaCO_3$ ($P = 0.000$), yeast extract ($P = 0.001$), and ammonium sulfate ($P = 0.034$) had significant effects on biobutanol production. However, $MgSO_4 \cdot 7H_2O$ ($P = 0.094$) and $FeSO_4 \cdot 7H_2O$ ($P = 0.820$) were negligible factors and had no effect on the biobutanol production (Tables 4 and 5).

The model interaction had a P-value of 0.00 and F-value of 122.88, which indicated that the model equation was reliable. The $R^2$ value was 0.9954, which indicated that the model contributed to 99.54% positive in the response of the variables content, and only less than 0.5% were not clarified. Meanwhile, the R-value was 0.9873, close to 1, which represented that the experimental and predicted values had a good correlation. Chen *et al.* (2009) state that the regression model has a very strong correlation when the $R^2$ value is greater than 0.9. The above analysis shows that the model was very fit to screen the significant nutrients that may affect the production of biobutanol. The experimental results that we obtained were very close to the predicted values (Table 3).

**Table 3.** Plackett-Burman Experimental Design and the Response Values (Experimental and Predicted)

| Run order | Parameters | | | | | | | Biobutanol (g/L) | |
|---|---|---|---|---|---|---|---|---|---|
| | *X1* | *X2* | *X3* | *X4* | *X5* | *X6* | *X7* | Observed | Predicted |
| 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 6.44 | 6.43 |
| 2 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 6.48 | 6.49 |
| 3 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 6.53 | 6.53 |
| 4 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 6.56 | 6.57 |
| 5 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 6.33 | 6.34 |
| 6 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 6.55 | 6.56 |
| 7 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 6.59 | 6.60 |
| 8 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 6.50 | 6.49 |
| 9 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 6.41 | 6.41 |
| 10 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 6.59 | 6.59 |
| 11 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 6.73 | 6.72 |
| 12 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 6.62 | 6.62 |

Furthermore, through the regression analysis the regression equation was established as follows:

$$\begin{aligned}\text{Butanol (g/L)} = {} & 6.53 + 0.0575 \times \text{Reducing Sugar} + \\ & 0.00750\ MgSO_4 \cdot 7H_2O + 0.0275\ \text{Yeast Extract} + \\ & 0.0592\ K_2HPO_4 + 0.00083\ FeSO4 \cdot 7H_2O + \\ & 0.0492\ CaCO_3 - 0.0108\ \text{Ammonium Sulfate} \end{aligned} \quad (3)$$

A Pareto plot can also reflect the effect of variables on biobutanol production. As shown in Fig. 1, the three most significant nutrients affecting biobutanol production from top to bottom were $K_2HPO_4$, reducing sugar, and $CaCO_3$.

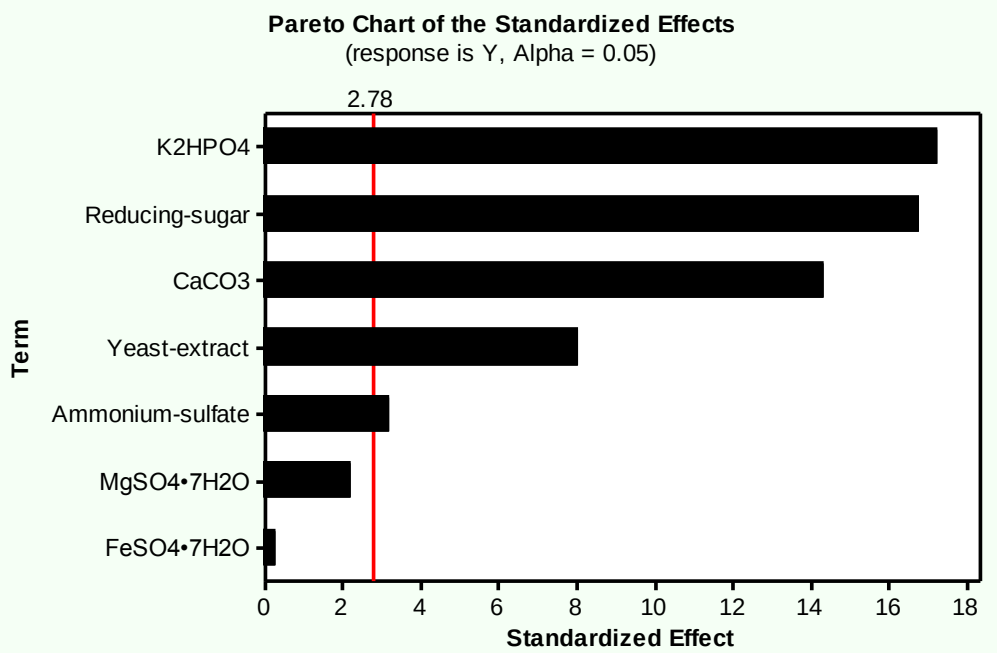


**Fig. 1.** Pareto chart of seven-factor standard effects on production of biobutanol

**Table 4.** Analysis of Main Effects of Factors for Plackett-Burman Design (Estimated Effects and Coefficients for Y (coded units)

| Term | Effect | Coef | SE Coef | T | P |
|---|---|---|---|---|---|
| Constant | | 6.52750 | 0.003436 | 1899.78 | 0.000 |
| Reducing sugar | 0.11500 | 0.05750 | 0.003436 | 16.73 | 0.000 |
| $MgSO_4 \cdot 7H_2O$ | 0.01500 | 0.00750 | 0.003436 | 2.18 | 0.094 |
| Yeast extract | 0.05500 | 0.02750 | 0.003436 | 8.00 | 0.001 |
| $K_2HPO_4$ | 0.11833 | 0.05917 | 0.003436 | 17.22 | 0.000 |
| $FeSO_4 \cdot 7H_2O$ | 0.00167 | 0.00083 | 0.003436 | 0.24 | 0.820 |
| $CaCO_3$ | 0.09833 | 0.04917 | 0.003436 | 14.31 | 0.000 |
| Ammonium sulfate | -0.02167 | -0.01083 | 0.003436 | -3.15 | 0.034 |

S = 0.0119024 PRESS = 0.0051
R-Sq = 99.54% R-Sq(pred) = 95.83% R-Sq(adj) = 98.73%

**Table 5.** Analysis of Variance for Plackett-Burman Design Affecting Biobutanol Production (coded units)

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Main Effects | 7 | 0.121858 | 0.121858 | 0.0174083 | 122.88 | 0.000 |
| Reducing sugar | 1 | 0.039675 | 0.039675 | 0.0396750 | 280.06 | 0.000 |
| $MgSO_4 \cdot 7H_2O$ | 1 | 0.000675 | 0.000675 | 0.0006750 | 4.76 | 0.094 |
| Yeast extract | 1 | 0.009075 | 0.009075 | 0.0090750 | 64.06 | 0.001 |
| $K_2HPO_4$ | 1 | 0.040008 | 0.040008 | 0.0400083 | 296.53 | 0.000 |
| $FeSO_4 \cdot 7H_2O$ | 1 | 0.000008 | 0.000008 | 0.0000083 | 0.06 | 0.820 |
| $CaCO_3$ | 1 | 0.029008 | 0.029008 | 0.0290083 | 204.76 | 0.000 |
| Ammonium sulfate | 1 | 0.001408 | 0.001408 | 0.0014083 | 9.94 | 0.034 |
| Residual Error | 4 | 0.000567 | 0.000567 | 0.0001417 | | |
| Total | 11 | 0.122425 | | | | |

In ABE fermentation, pH was considered to have the main effect on biobutanol production (Salleh *et al.* 2008). The pH was decreased during the acidogenic phase due to the production of butyric and acetic acids, and then it was increased in the solventogenic phase due to the re-assimilation of acids (Al-Shorgani *et al.* 2013). It was reported that pH is responsible for the initiation of the solventogenic enzymes (Li *et al.* 2014). $K_2HPO_4$ is a kind of phosphate commonly used for a buffer, especially in a bacteriological culture medium, which has a buffering effect that can maintain the pH during fermentation. $CaCO_3$ also had a notable positive effect on pH ascribed to the buffering effect of carbonate, which could be increased. $Ca^{2+}$ was found to have the ability to promote the production of biobutanol (Isar and Rangaswamy 2012; Han *et al*. 2013). Additionally, Richmond *et al.* (2011) found that $CaCO_3$ could increase the *Clostridium*'s tolerance against the accumulation of biobutanol due to the presence of bivalent ions ($Ca^{2+}$), which may also enhance the stability of membrane proteins.

As a carbon source, the concentration of reducing sugar was the second most significant factor affecting biobutanol production in this study. The presence of an abundant amount of fermentable sugars in the fermentation medium is essential for the maintenance of ABE production (Jin *et al.* 2011). Carbon source is the material base of maintaining microorganisms or cells to normal growth, division, and reproduction, which can provide cellular carbon frame, the energy for cell life activities, and the carbon frame of synthetic products. In the acidogenesis phase of *Clostridium* metabolism, cells using carbon source to generate ATP and NHDP, all of which are used to maintain the activity of cells provide the energy for the formation of metabolites in the solventogenesis phase. Yeast extract and ammonium sulfate were used as a nitrogen source for cell growth of microorganisms and to promote the fermentation processes (Ranjan *et al.* 2013). Abd-Alla and Elsadek El-Enany (2012) found that yeast extract could significantly increase biobutanol production due to having a strong effect on sugar utilization during fermentation. Chua *et al*. (2013) also found that increasing the yeast extract addition can enhance biobutanol production.

The PBD results of this study suggest that higher $K_2HPO_4$ concentration, higher reduced sugar concentration, higher $CaCO_3$ concentration, and lower ammonium sulfate concentration can accelerate biobutanol production by using a mutant of *Clostridium saccharobutylicum* in shake-flask fermentation. Moreover, an interaction is probable among the significant variables exhibited by the analysis, which will affect biobutanol production. Hence, in order to indicate the interactions among these significant factors and

consequently improve biobutanol fermentation by the mutant strain of *Clostridium saccharobutylicum*, using a response surface methodology (RSM) to further optimize the medium was needed.

## Box-Behnken Design for Optimization of Biobutanol Production

In this study, RSM based on a Box-Behnken design was implemented to optimize the three most important variables of $K_2HPO_4$ ($X_1$), reducing sugar concentrations in the poplar wood hydrolysate ($X_2$), and $CaCO_3$ ($X_3$) for biobutanol production by a mutant strain of *C. saccharobutylicum*, and the experimental matrix is presented in Table 6. The statistical significance of the model was analyzed using ANOVA, as shown in Table 7. The model's F-value of 80.34 and p-value of less than 0.0001 imply the model was significant, and the "Lack of Fit F-value" of 0.57 implies that the Lack of Fit was not significant, relative to the pure error. The most significant factors affecting the biobutanol yield were $K_2HPO_4$ ($p < 0.0001$) followed by $CaCO_3$ ($p < 0.0001$) and reducing sugar concentrations ($p = 0.0003$). Results from the Box-Behnken design output show that the optimum values of $K_2HPO_4$, $CaCO_3$, and reducing sugar concentrations were 1.35 g/L, 4.65 g/L, and 44.53 g/L, respectively. The predicted value of biobutanol production was 7.59 g/L. The second-order model equation of actual variables and the predicted response was fitted to the data as follows:

$$\begin{aligned}\text{Butanol (g/L)} = {} & 7.35 + 0.35 \times K_2HPO_4 + 0.12 \times CaCO_3 + \\ & 0.26 \times \text{Reducing Sugar} - 0.045 \times K_2HPO_4 \times CaCO_3 - \\ & 0.11 \times K_2HPO_4 \times \text{Reducing Sugar} - \\ & (7.500\ \text{E-003}) \times CaCO_3 \times \text{Reducing Sugar} - \\ & 0.18 \times K_2HPO_4^2 - 0.093 \times CaCO_3^2 - \\ & 0.14 \times \text{Reducing Sugar}^2 \end{aligned} \quad (4)$$

With biobutanol production as the response, the three-dimensional response surfaces are presented in Fig. 2. These figures reflect the relative effects of two variables while keeping the third factor constant. In Fig. 2(A), the interaction of $K_2HPO_4$ and reducing sugar concentrations in poplar wood hydrolysate is represented. The variation of $K_2HPO_4$ was noticeably more important than reducing sugar concentrations in biobutanol production.

Figure 2(B) shows that the interactive effect between $K_2HPO_4$ and reducing sugar concentrations had a symmetrical mound shape with an axis of symmetry parallel to the diagonal, indicating the significance of these two variables. The variation of $CaCO_3$ and reducing sugar concentrations influenced biobutanol production, as shown in Fig. 2(C). The variation of $CaCO_3$ was relatively more important than that of reducing sugar concentrations relative to biobutanol production. The order of importance of the three variables on biobutanol production was: $K_2HPO_4$ > $CaCO_3$ > reducing sugar concentrations.

In order to verify the validity of the optimization strategy, three replicate experiments were conducted under the optimized conditions described above. The biobutanol production was obtained at 7.61 ± 0.15 g/L, which is almost the same as that predicted value (7.59 g/L) through RSM. This validated that the RSM approach was effective for optimizing the nutritious components for the biobutanol fermentation.

**Table 6.** Result of the Response Surface Test

| Run | $X_1$ | $X_2$ | $X_3$ | Y(g/L) |
|---|---|---|---|---|
| 1 | 0 | 1 | -1 | 6.98 |
| 2 | 1 | 0 | 1 | 7.54 |
| 3 | -1 | 0 | -1 | 6.31 |
| 4 | -1 | 1 | 0 | 6.91 |
| 5 | -1 | -1 | 0 | 6.54 |
| 6 | 0 | 0 | 0 | 7.32 |
| 7 | 1 | -1 | 0 | 7.34 |
| 8 | 0 | 1 | 1 | 7.46 |
| 9 | 0 | 0 | 0 | 7.42 |
| 10 | 1 | 1 | 0 | 7.53 |
| 11 | 0 | 0 | 0 | 7.29 |
| 12 | -1 | 0 | 1 | 7.08 |
| 13 | 0 | -1 | -1 | 6.76 |
| 14 | 0 | 0 | 0 | 7.41 |
| 15 | 0 | -1 | 1 | 7.27 |
| 16 | 1 | 0 | -1 | 7.19 |
| 17 | 0 | 0 | 0 | 7.33 |

**Table 7.** ANOVA for Response Surface Quadratic Model of Biobutanol Production

| Source | Sum of Squares | df | Mean Square | F-Value | p-value Prob > F | |
|---|---|---|---|---|---|---|
| Model | 1.97 | 9 | 0.22 | 80.34 | < 0.0001 | significant |
| $X_1$ | 0.95 | 1 | 0.95 | 349.98 | < 0.0001 | |
| $X_2$ | 0.12 | 1 | 0.12 | 43.23 | 0.0003 | |
| $X_3$ | 0.56 | 1 | 0.56 | 204.55 | < 0.0001 | |
| $X_1X_2$ | 8.100E-003 | 1 | 8.100E-003 | 2.98 | 0.1281 | |
| $X_1X_3$ | 0.044 | 1 | 0.044 | 16.21 | < 0.0050 | |
| $X_2X_3$ | 2.250E-004 | 1 | 2.250E-004 | 0.083 | 0.7820 | |
| $X_1^2$ | 0.14 | 1 | 0.14 | 50.56 | 0.0002 | |
| $X_2^2$ | 0.037 | 1 | 0.037 | 13.46 | 0.0080 | |
| $X_3^2$ | 0.086 | 1 | 0.086 | 31.76 | 0.0008 | |
| Residual | 0.019 | 7 | 2.721E-003 | | | |
| Lack of Fit | 5.725E-003 | 3 | 1.908E-003 | 0.57 | 0.6623 | not significant |
| Pure Error | 0.013 | 4 | 3.330E-003 | | | |
| Cor Total | 1.99 | 16 | | | | |

Note: $R^2$ = 0.9904, Adj $R^2$ = 0.9781, Pred $R^2$= 0.9434

## Identification of Optimal Fermentation Conditions

In order to achieve maximum biobutanol production, on the basis of a single factor experiment, it was necessary to carry out optimization by orthogonal combination under multi-factor test conditions. Three factors and three levels were used in the orthogonal experiment. The experimental combinations and biobutanol production for each column are shown in Table 8. The orthogonal experiment results were analyzed by the SPSS statistical package. Between-subjects effects and estimated marginal means of the three variables are presented in Tables 9 and 10.

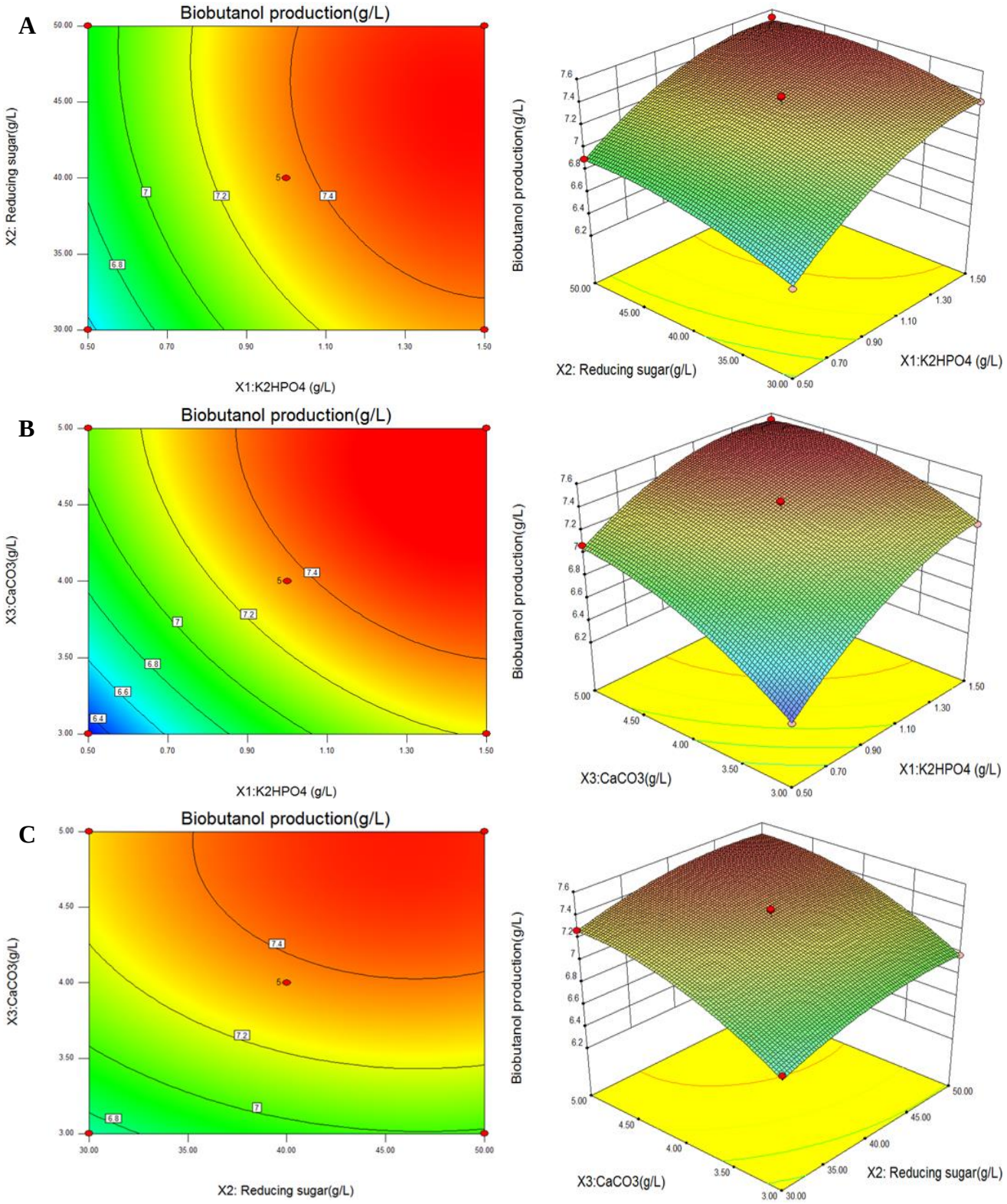


**Fig. 2.** Response surface for biobutanol production as a result of the interaction between $K_2HPO_4$ and reducing sugar concentration (A), the interaction between $K_2HPO_4$ and $CaCO_3$ (B), and the interaction between $CaCO_3$ and reducing sugar concentration (C)

In Table 9, the type III sum of squares and the P-value of the three variables show that initial pH had the largest effect on biobutanol production, temperature had the second largest effect, and inoculum size had the smallest effect on biobutanol production. The best levels for each variable were an initial pH of 6.5, fermentation temperature of 36 °C, and an inoculum size of 9% through the estimated marginal means analysis in Table 10.

Under the optimal fermentation medium compositions and fermentation conditions, validation experiments were carried out in 250 mL screw-capped bottles and 3 L fermentation tanks. Biobutanol production was 8.30 ± 0.12 g/L and 8.41 ± 0.20 g/L, respectively. The results showed that optimizing medium components and fermentation conditions made it possible to effectively increase biobutanol production using a mutant of *Clostridium saccharobutylicum*, such that the biobutanol yield was 0.21 g/g. Ibrahim *et al.* (2012) utilized oil palm empty fruit bunch hydrolysate as substrate to ferment, and obtained the biobutanol yield of 0.13g/g under optimum conditions. Al-Shorgani *et al.* (2013) adopted Plackett-Burman Design to optimize the medium composition for biobutanol production; they obtained a biobutanol yield of 0.22 g/g glucose. Wang and Blaschek (2011), who used tropical maize stalk juice for the fermentation, obtained a biobutanol yield of 0.27g/g under optimized experimental conditions. Additionally, the work showed that using biostatistical methods (such as Plackett-Burman design, Box-Behnken designs and orthogonal design) could substantially improve the biobutanol fermentation performance.

**Table 8.** Orthogonal Test for Fermentation Condition Optimization of Biobutanol Production

| Initial pH | Temperature (°C) | Inoculum size (%) | STATUS | CARD | Biobutanol (g/L) |
|---|---|---|---|---|---|
| 7 | 38 | 7 | 0 | 1 | 5.41 |
| 6 | 36 | 11 | 0 | 2 | 7.23 |
| 7 | 34 | 11 | 0 | 3 | 6.68 |
| 6 | 38 | 9 | 0 | 4 | 6.54 |
| 6.5 | 38 | 11 | 0 | 5 | 7.13 |
| 7 | 36 | 9 | 0 | 6 | 7.16 |
| 6.5 | 36 | 7 | 0 | 7 | 8.02 |
| 6.5 | 34 | 9 | 0 | 8 | 8.27 |
| 6 | 34 | 7 | 0 | 9 | 6.86 |

**Table 9.** Orthogonal Tests of Between Subjects Effects for Biobutanol Production

| Source | Type III Sum of Squares | df | Mean Squares | F | P(Sig.) |
|---|---|---|---|---|---|
| Corrected Model | 5.581[a] | 6 | 0.930 | 95.893 | 0.010 |
| Intercept | 445.210 | 1 | 445.210 | 45897.938 | 0.000 |
| Initial pH | 3.009 | 2 | 1.504 | 155.082 | 0.006 |
| Temperature | 2.100 | 2 | 1.050 | 108.258 | 0.009 |
| Inoculum size | 0.472 | 2 | 0.236 | 24.340 | 0.039 |
| Error | 0.019 | 2 | 0.010 | | |
| Total | 450.810 | 9 | | | |
| Corrected Total | 5.600 | 8 | | | |
| a. R Squared =0.997（Adjusted R Squared =0.986） | | | | | |

**Table 10.** Orthogonal Tests of Estimated Marginal Means for Biobutanol Production

| Initial pH | **mean** | Std. Error | confidence interval | |
|---|---|---|---|---|
| | | | lower bound | upper bound |
| 6.0 | 6.877 | 0.057 | 6.632 | 7.121 |
| 6.5 | 7.807 | 0.057 | 7.562 | 8.051 |
| 7.0 | 6.417 | 0.057 | 6.172 | 6.661 |
| Temperature | **mean** | Std. Error | confidence interval | |
| | | | lower bound | upper bound |
| 34 | 7.270 | 0.057 | 7.025 | 7.515 |
| 36 | 7.470 | 0.057 | 7.225 | 7.715 |
| 38 | 6.360 | 0.057 | 6.115 | 6.605 |
| Inoculum size | **mean** | Std. Error | confidence interval | |
| | | | lower bound | upper bound |
| 7 | 6.763 | 0.057 | 6.519 | 7.008 |
| 9 | 7.323 | 0.057 | 7.079 | 7.568 |
| 11 | 7.013 | 0.057 | 6.769 | 7.258 |

ABE fermentation is characterized by biphasic fermentation. It has long been believed that the pH of the fermentation medium is a key factor affecting the solvent production because the rapid formation of organic acid causes the pH to decrease in the acidogenesis stage. However, when the pH is below a ‘‘break point’’ of 4.5, acids may not reassimilate and transform to biobutanol and acetone in solventogenesis (Lee *et al*. 2008). Therefore, the strength of the buffering capacity in the fermentation medium may have a crucial effect on biobutanol production. $K_2HPO_4$ and $CaCO_3$ are understood to be effective buffers in biobutanol production. Also, $Ca^{2+}$ could combine with some inhibitors of hydrolysate such as furan (Purwadi *et al*. 2004). Moreover, when the pH is uncontrolled in ABE fermentation processes, to assure the switch to solventogenesis and high biobutanol yield, an appropriate initial pH is extremely important.

## CONCLUSIONS

1. The significant medium nutrient compositions ($K_2HPO_4$, reducing sugar concentration, and $CaCO_3$), which influenced the biobutanol production by a mutant of *Clostridium*

*saccharobutylicum*, were successfully screened using the Plackett-Burman design. Furthermore, using Box-Behnken design analysis suggested that the most suitable levels of variables were 44.53 g/L of reducing sugar concentration from poplar wood hydrolysate, 1.36 g/L of $K_2HPO_4$, and 4.65 g/L of $CaCO_3$ with a predicted biobutanol production of 7.59 g/L, and an actual biobutanol production of 7.61 ± 0.15 g/L through validation.

2. An orthogonal design, used for optimizing the biobutanol fermentation condition, suggested that initial pH, fermentation temperature, and inoculum size were all significant factors affecting biobutanol production. The best combination was an initial pH of 6.5, fermentation temperature of 36 °C, and inoculum size of 9%. Verification results showed that the biobutanol production was 8.30 ± 0.12g/L and 8.41 ± 0.20 g/L, respectively, in 250 mL screw-capped bottles and 3 L fermentation tanks under the optimal fermentation medium compositions and fermentation conditions.

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