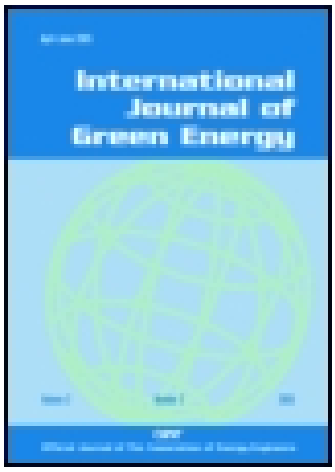

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Response Surface Methodology for Biobutanol Optimization Using Locally Isolated Clostridium acetobutylicum YM1

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Short Title: RSM FOR BIOBUTANOL OPTIMIZATION BY *Clostridium* YM1

Response Surface Methodology for Biobutanol Optimization Using Locally Isolated *Clostridium acetobutylicum* YM1

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Abstract

In this study, response surface methodology (RSM) was applied to optimize and investigate the ability of yeast extract, $CaCO_3$, $MgSO_4$ and K_2HPO_4 to maximize biobutanol production by a novel local isolate of *Clostridium acetobutylicum* YM1. A central composite design was applied as the experimental design, and analysis of variance (ANOVA) was used to analyze the experimental data. A quadratic polynomial equation was obtained for biobutanol production by multiple regression analysis. ANOVA analysis showed that the model was significant ($p < 0.0001$), and the yeast extract, $CaCO_3$ and $MgSO_4$ concentrations had a significant effect on biobutanol production. However, K_2HPO_4 did not have a significant effect on biobutanol

production. The estimated optimum combinations for biobutanol production using *C. acetobutylicum* YM1 were 2 g/L yeast extract, 6 g/L $CaCO_3$, 0.1 g/L $MgSO_4$ and 1.1 g/L K_2HPO_4. Subsequently, the model was validated through use of the estimated optimum conditions, which confirmed the model validity and 13.67 g/L of biobutanol was produced.

Keywords: Biobutanol; *Clostridium acetobutylicum* YM1; Medium optimization; Response surface methodology; Renewable energy.

INTRODUCTION

Butanol is currently considered to be an alternative and renewable biofuel due to the expected exhaustion of conventional oil and growing demand for energy as well as increasing global oil price. Butanol has many advantageous properties as a biofuel. Compared to ethanol, butanol has a higher energy content, is less corrosive, has a lower vapor pressure, can be used directly or in combination with gasoline, can be shipped through existing infrastructure and can be used in existing engines without any modification (Dürre 2007, 2008, Lee et al. 2008). Moreover, butanol mimics the properties of gasoline more closely than other biofuels, such as ethanol. Butanol may be the next-generation biofuel that replaces gasoline (Tashiro and Sonomoto 2010).

Biologically, biobutanol is produced via anaerobic acetone-butanol-ethanol (ABE) fermentation using many solvent-producing clostridia strains such as *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* and *C. pasteurianum* (Formanek, Mackie, and Blaschek 1997, Qureshi and Maddox 1992). Previous studies conducted on ABE fermentation provided information used to improve biobutanol production, including screening for new microbes,

developing new fermentation systems, developing saccharification or pretreatment methods for lignocellulosic materials that can be used as cheap substrates in ABE fermentation, using molecular tools to improve the selectivity of biobutanol production and improving recovery systems and microbial metabolic engineering. However, existing biobutanol fermentation processes still have many facets that make this process not economical, including the low biobutanol productivity caused by the effects of biobutanol accumulation during fermentation, the price of the substrate and the recovery cost of biobutanol. Screening for new biobutanol-producing microbes that have the ability to generate higher amounts of biobutanol and can tolerate high concentrations of solvent is one solution to overcoming the low productivity of biobutanol production. Moreover, maximizing the production of biobutanol by optimization of medium and fermentation conditions is vital.

Response surface methodology (RSM) is a useful statistical optimization design that has successfully been used in many biological and chemical processes (Vishwanatha, Rao, and Singh 2010, Makareviciene et al. 2013, Reyhani and Zilouei 2012). RSM creates an experimental design with the minimum number of experiments and generates a model that can predict the interaction and correlation between a set of independent variables and observed results, thus providing optimized conditions (Bezerra et al. 2008, Zheng et al. 2008, Borjan et al. 2011).

Solvent-producing *Clostridia* are auxotrophic and many media were used in ABE fermentation including reinforced clostridial medium (RCM), tryptone yeast-extract acetate medium (TYA), and P2 medium while some strains have special nutrients requirements to enhance the growth and biobutanol production. Hence in this study, medium was optimized to improve biobutanol

production. Yeast extract, $CaCO_3$, K_2HPO_4 and $MgSO_4$ were selected for biobutanol optimization by response surface methodology (RSM) based on our previous screening study using the Plackett-Burman design (Al-Shorgani et al. 2013) which showed that these nutritional factors have the main effect on butanol production using *C. acetobutylicum* strain YM1.

The aim of this study was to optimize biobutanol production using RSM with central composite design (CCD) by investigating the effects of some of the medium components, including yeast extract, $CaCO_3$, K_2HPO_4 and $MgSO_4$, as well as their interactions on biobutanol production using a new isolate of solvent-producing *C. acetobutylicum* YM1 isolated from local soil.

MATERIALS AND METHODS

Microorganism

C. acetobutylicum YM1 (GenBank accession No. KC969670) isolated from soil was used in this study, and the culture was maintained on 50% glycerol as a spore suspension at -30 °C. The inoculum was prepared by activating the spores in tryptone yeast extract acetate medium (TYA), which consisted of 20 g/L glucose, 6 g/L tryptone, 2 g/L yeast extract, 3 g/L ammonium acetate, 0.5 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, and 0.01 g/L $FeSO_4.7H_2O$.

Growth Medium and Culture Conditions

The fermentation medium that was used consisted of the following (g/L): glucose 50; tryptone 6; ammonium acetate 3; KH_2PO_4 0.75; $FeSO_4$ 0.05; and NaCl 0.1. The concentrations of yeast

extract, $CaCO_3$, $MgSO_4$ and K_2HPO_4 were varied according to the requirements of each cultivation experiment as designed by the CCD (Table 2). The initial pH of all fermentation experiments was adjusted initially to 6.3 with 3 M NaOH or 3 M HCl. The fermentation experiments were conducted in 250 mL Scott Duran bottles, with a working volume of 100 mL, which were inoculated aseptically with a 10% fresh culture of *C. acetobutylicum* YM1 and incubated at 30 °C.

Analysis

Samples of the fermentation broth were centrifuged at 7000 rpm for 5 min before analysis. ABE and butyric and acetic acids were analyzed by gas chromatography (7890A GC-System, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and 30 m capillary column (Equity1^{TM}; 30 m × 0.32 mm × 1.0 µm; Supelco Co., USA). The oven temperature was programmed to increase from 40 to 130°C at a rate of 8°C/min. The injector and detector temperatures were set at 250 and 280°C, respectively. Helium was the carrier gas and was set at a flow rate of 1.5 mL/min (Al-Shorgani et al. 2012).

Steepest Ascent Design

Screening by Plackett-Burman design identified the significant nutrients involved in biobutanol production by *C. acetobutylicum* YM1. These factors included peptone, yeast extract, $CaCO_3$, $MgSO_4$ and K_2HPO_4. To optimize biobutanol production, a steepest ascent experiments were conducted (Table 1). The range of the selected variables was varied from the initial concentration at the center of the factorial design (Plackett-Burman design) and ended when no further

enhancement in biobutanol production could be achieved (Wang and Wan 2009). The data generated from steepest ascent was used for further optimization by RSM.

Experimental Design and Statistical Analysis

Four independent variables were chosen for optimization according to the screening study, which was conducted using a Plackett-Burman design, as described previously (Al-Shorgani et al. 2013). These variables were the yeast extract concentration, $CaCO_3$ concentration, $MgSO_4$ concentration and K_2HPO_4 concentration, and the biobutanol concentration was taken as the response when designing the experiments using Design of Expert Software (DOE; version 6.0.10, Stat Ease, USA) with RSM.

The RSM with CCD design resulted in 30 experiments with different combinations of the four independent variables. The experiments were carried out in batch culture of *Clostridium acetobutylicum* YM1 isolate in 250 mL Duran Scott bottles, with a working volume of 100 mL. The experimental conditions were 30 °C incubation temperature, 10% inoculum and anaerobic conditions. The ranges for the experimental design were selected based on the steepest ascent design and our previous study (Al-Shorgani et al. 2013). Statistical analysis, using an ANOVA with the DOE software, was used to estimate the optimal culture conditions for maximum biobutanol production.

The coefficients in the second-order polynomial (Eq. 1) were calculated by multiple regression analysis of the experimentally obtained results.

$$Y = b'_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} b_{ij} X_i X_j \qquad \text{(Eq.1)}$$

where Y is the predicted response, b'_0 is the constant coefficient, bi is the linear coefficient, b_{ij} is the interaction coefficient, b_{ii} is the quadratic coefficient, and X_i and X_j are coded values.

The biobutanol production in batch fermentation under optimum conditions was then compared with the predicted value obtained from the model.

RESULTS AND DISCUSSION

Steepest Ascent Design

According to the analysis of the prescreening design using a Plackett-Burman design, as reported previously (Al-Shorgani et al. 2013), the steepest ascent design was used to alter and narrow the ranges of the most important factors affecting biobutanol production. Here, the yeast extract, peptone, $CaCO_3$, $MgSO_4$ and K_2HPO_4 concentrations used are presented in Table 1.

Peptone does not have a significant effect on biobutanol production, and to minimize the medium compositions, peptone was excluded from the next set of experiments (Table 1; runs 8 and 9). The results from steepest ascent showed that maximum biobutanol production can be obtained the conditions in run 5 and 6 (Table 1). The maximum biobutanol production (10.51 g/L) was obtained from 4 g/L yeast extract, 5 g/L $CaCO_3$, 0.5 g/L $MgSO_4$ and 1 g/L K_2HPO_4 (Table 1; run 6). It is clear that increasing the concentration of $CaCO_3$ leads to an increase in biobutanol production. Moreover, increasing the yeast extract up to 4 g/L enhanced biobutanol

production (Table1; run 6),but more than 4 g/L of yeast extract decreased biobutanol production (Table 1; run 7). The information from the steepest ascent design was used for the next optimization using RSM with CCD design.

Central Composite Optimization Design

Conventional medium optimization by the traditional one-factor-at-a-time method does lead to enhanced biobutanol production,;however, this method takes a great deal of time and cannot show potential interactions between the medium components (Souza, Flôres, and Ayub 2006).

RSM is suitable for optimizing multiple variables, saving time and estimating the relationship between more than one variable and a given response (Liu et al. 2005). Therefore, RSM was used as a statistical tool in medium optimization to obtain optimal medium components for maximizing the production of biobutanol.

In the present study, RSM based on a central composite design was applied to optimize the medium compositions for biobutanol production by a new strain of *C. acetobutylicum* YM1, and the experimental matrix is presented in Table 2. The levels of each factor were selected based on the Plackett-Burman design (Al-Shorgani et al. 2013) and the steepest ascent, as discussed above. The biobutanol production results (experimental and predicted) obtained from 30 experiments designed by CCD are shown in Table 2. The experimental biobutanol production results ranged from 2.22 to 12.55 g/L, which were close to the predicted values.

In all of the tested models, linear, two-factor interactions and quadratic models for biobutanol production indicate the adequacy of the models, and all these models were strongly significant,

with an F-value of $p< 0.0001$ (Table 3). This means that the confidence levels were greater than 99.99%. Meanwhile, the F-test had a very low error probability ($P > F= 0.0001$) and exhibited very high significance values for these regression models. An ANOVA was used to test the adequacy of the full quadratic model of biobutanol production, and the data from the ANOVA is presented in Table 4.

The influence of the yeast extract, $CaCO_3$, $MgSO_4$ and K_2HPO_4 concentrations on biobutanol production was tested using an ANOVA. The analysis of quadratic regression using ANOVA was applied to estimate the significance of the model coefficients. The significance of each coefficient was indicated by the *p* values, which also demonstrate the interaction strength between each independent variable. The *p* value of each variable of the model showed that yeast extract, $CaCO_3$ and $MgSO_4$ have significant effects ($p < 0.05$) on biobutanol production, and only K_2HPO_4 did not have a significant effect (Table 4). The function of K_2HPO_4 and KH_2PO_4 in the medium are as phosphate sources as well as buffering agents which is important in biobutanol fermentation due to the biphasic fermentation behavior. Presence of KH_2PO_4 (0.75 g/L) in the medium beside K_2HPO_4 may be did not show the significant effect on biobutanol production. Moreover, the non significant effect of K_2HPO_4 can be attributed also to the studied range which varied between 0.1 and 1.19 g/L.

The interaction effects between yeast extract and $CaCO_3$ as well as the interactive effects between $CaCO_3$ and $MgSO_4$ were not significant, whereas the interaction effects of any other pair of variables were significant (Table 4).

The model equation in actual terms of variables and the predicted response (biobutanol production) was as follows:

Butanol (g/L) = 9.05 - 0.05 × Yeast Extract – 0.03 × $CaCO_3$ + 1.50 × $MgSO_4$ – 0.03 × K_2HPO_4 - 0.07 × Yeast Extract^2 + 0.08 × $CaCO_3^2$ + 0.95 × $MgSO_4^2$ – 0.86 × $K_2HPO_4^2$ - 0.07 × Yeast Extract × $CaCO_3$ - 0.71 × Yeast Extract × $MgSO_4$ + 0.39 × Yeast Extract × K_2HPO_4 - 0.20 × $CaCO_3$ × $MgSO_4$ + 0.35 × $CaCO_3$ × K_2HPO_4 - 2.05 × $MgSO_4$ × K_2HPO_4 (Eq. 2).

The coefficient of determination (R^2) was 0.954, which means that the model can explain 95.4% of the variability of the response variable, and only 4.4% of variables cannot be represented by this model. The lack of fit of the model was 0.2055, which confirmed the fitness of the model and verified that the model was able to sufficiently describe the data in the experimental region.

The adequate precision value measures the signal to noise ratio, and a value greater than 4 is desirable for the appropriate fitness of the quadratic model. As shown by the results in Table 4, the adequate precision value of 18.31 indicates desirable fitness.

Effects of Variables on Biobutanol Production

The three-dimensional (3D) response surface was plotted to study the interaction among the four factors investigated and to visualize the combined effects of the variables on biobutanol production (Figure 1). In the 3D graphs, the effect of two variables was plotted, while another variable was constant at the center point. The constant values were 4 g/L of yeast extract, 3.13 g/L of $CaCO_3$, 0.64 g/L of $MgSO_4$, and 0.64 g/L of K_2HPO_4, as shown in Table 2.

The combined effects of yeast extract and $CaCO_3$showed that biobutanol production increased with an increase in $CaCO_3$ and decrease in the yeast extract concentration (Figure 1 a). The effects of yeast extract and $MgSO_4$ also led to an increase biobutanol production with a decrease in yeast extract and increase in $MgSO_4$ concentration (Figure 1 b). The 3D graph shows that the maximum biobutanol production was obtained with the highest $CaCO_3$ concentration; therefore, the most significant factor affecting biobutanol production is $CaCO_3$. The interaction effect between $MgSO_4$ and K_2HPO_4 demonstrated that biobutanol production increased with an increase in K_2HPO_4at moderate concentrations of $MgSO_4$ (Figure 1 c). The combinatory effects of K_2HPO_4 and yeast extract for biobutanol production are presented in Figure 1 d. It can be observed that the production of biobutanol increased with increasing K_2HPO_4 concentration and decreasing yeast extract concentration. In this 3D plot, the highest concentration of biobutanol was obtained with 2.45 g/L yeast extract and 1.03 g/L K_2HPO_4 (Figure 1 c).

Yeast extract is required for microbial growth. The effect of yeast extract was attributed to the degradation of the amino acids in yeast extract, which is the major source for growth (Zhang and Wiegel 1990). It is clear that yeast extract is an important factor in biobutanol fermentation by solvent-producing *Clostridium* strains. This study confirmed the importance of yeast extract on biobutanol production and bacterial growth. Increasing yeast extract beyond 4 g/L has a negative effect on the production of biobutanol by *C. acetobutylicum* YM1.

The p value of the yeast extract concentration was $p<0.0001$, which demonstrated that the yeast extract concentration had a significant effect on biobutanol production; an increase in the concentration of yeast extract decreased the production of biobutanol. In contrast, Razak et al.

found that yeast extract was not significant factor in biobutanol production from oil palm decanter cake hydrolysate (Razak et al. 2013). However, our results are in agreement with those reported by Madihah et al. (2001) and Ibrahim et al. (2012) who found that using a high concentration of yeast extract resulted in a decrease in the production of biobutanol. Moreover, the yeast extract concentration has reported as a significant factor in biobutanol production, and it has an effect on bacterial growth (Kong et al. 2004, Lin et al. 2011, Abd-Alla and El-Enany 2012).

C. saccharoperbutylacetonicum ATCC 27021 was found to prefer yeast extract as a nitrogen source when glucose was the carbon source in hydrogen production (Ferchichi et al. 2005). For *C. acetobutylicum* in solvent-production medium, Yerushalmi and Volesky (1987) reported that moderately high concentrations of yeast extract (7.5 g/L) and ammonium sulfate (3 to 6 g/L) as nitrogen sources were required for normal ABE generation and cellular growth.

The results of this study also demonstrated that $CaCO_3$ significantly affected biobutanol production ($p < 0.0001$). It was reported that calcium carbonate increases growth of *C. beijerinckii* NCIMB 8052, substrate consumption, and ABE production (Han et al. 2013). Moreover, carbonate has a buffering effect on medium fermentation (Fouad, Abou-Zeid, and Yassein 1976), and it was found that excess $CaCO_3$ in the medium leads to enhanced biobutanol production, which may be due to biobutanol tolerance and improved xylose utilization by *C. acetobutylicum* (Kanouni et al. 1998).

In a study by Richmond et al. (2011) investigating the effect of $CaCO_3$ on biobutanol production and growth using two *Clostridium* species (*C. acetobutylicum* ATCC 824 and *C. beijerinckii*

260), it was found that addition of $CaCO_3$ significantly enhanced the growth of and total ABE production by *C. acetobutylicum* ATCC 824 and *C. beijerinckii* 260. The ABE produced 31% to 46% more biobutanol, respectively, than the control medium without $CaCO_3$ addition. In addition, the same study revealed that supplementation with $CaCO_3$ increased biobutanol tolerance by more than 40% (Richmond, Han, and Ezeji 2011). Raganati et al. (2012) reported that the addition of $CaCO_3$ improves the overall biobutanol fermentation performance, which leads to accelerating the sugar utilization and rate of sugar conversion, maximizing the acid production and enhancing solvent generation.

The addition of Mg^{2+} ion to biobutanol fermentation medium showed an effect similar to that of carbonate, which improved biobutanol production and substrate consumption. It was suggested that the excess of the Mg^{2+} bivalent ion created relative stability for the membrane proteins (Kanouni et al. 1998). Putra (2009) found that supplementing medium with $MgSO_4$ strongly enhanced the production of biobutanol and growth of *C. beijerinckii.* On the other hand, the addition of 6 g/L $MgSO_4$ enhanced the growth of *C. beijerinckii* but not biobutanol production, suggesting that $MgSO_4$ has an effect on biobutanol production through its positive effect on bacterial growth (Putra 2009).

Based on the regression analysis of the model equation, the optimum levels of the variables were estimated. The optimum conditions were 2 g/L of yeast extract, 6 g/L of $CaCO_3$, 0.1 g/L of $MgSO_4$ and 1.1 g/L of K_2HPO_4. The maximum predicted biobutanol production was 12.6 g/L according to the model equation (Eq. 2).

Model Verification

To verify the predicted biobutanol production, a verification batch culture was carried out with the estimated optimum conditions of yeast extract (2 g/L), $CaCO_3$ (6 g/L), $MgSO_4$ (0.1 g/L) and K_2HPO_4 (1.1 g/L) with *C. acetobutylicum* YM1. The obtained biobutanol production from optimized medium was 13.67 g/L, which was 1.5 times higher than that of TYA medium (containing 50 g/L glucose) and agreed with the predicted value (12.6 g/L). The optimized medium consisted of 50 g/L glucose, 6 g/L tryptone, 3 g/L ammonium acetate, 0.75 g/L KH_2PO_4, 0.05 g/L $FeSO_4$, 0.1 g/L NaCl, 2 g/L yeast extract, 6 g/L $CaCO_3$, 0.1 g/L $MgSO_4$ and 1.1 g/L K_2HPO_4.

CONCLUSIONS

Medium optimization for biobutanol production using RSM was successfully conducted for predicting and understanding the interaction effects between the selected factors using CCD. The quadratic model shows that yeast extract, $CaCO_3$ and $MgSO_4$ had a significant effect on biobutanol production, while K_2HPO_4 did not. The optimum conditions to maximize biobutanol production are as follows: 2 g/L yeast extract, 6 g/L $CaCO_3$, 0.1 g/L $MgSO_4$ and 1.1 g/L K_2HPO_4. Validation the model resulted in 13.67 g/L of butanol, which is in agreement to the predicted value.

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Figure 1 Response surface plots illustrating the interaction effects of $CaCO_3$ and yeast extract (a), $MgSO_4$ and yeast extract (b), K_2HPO_4 and $MgSO_4$ (c), and K_2HPO_4 and yeast extract (d) on biobutanol production.

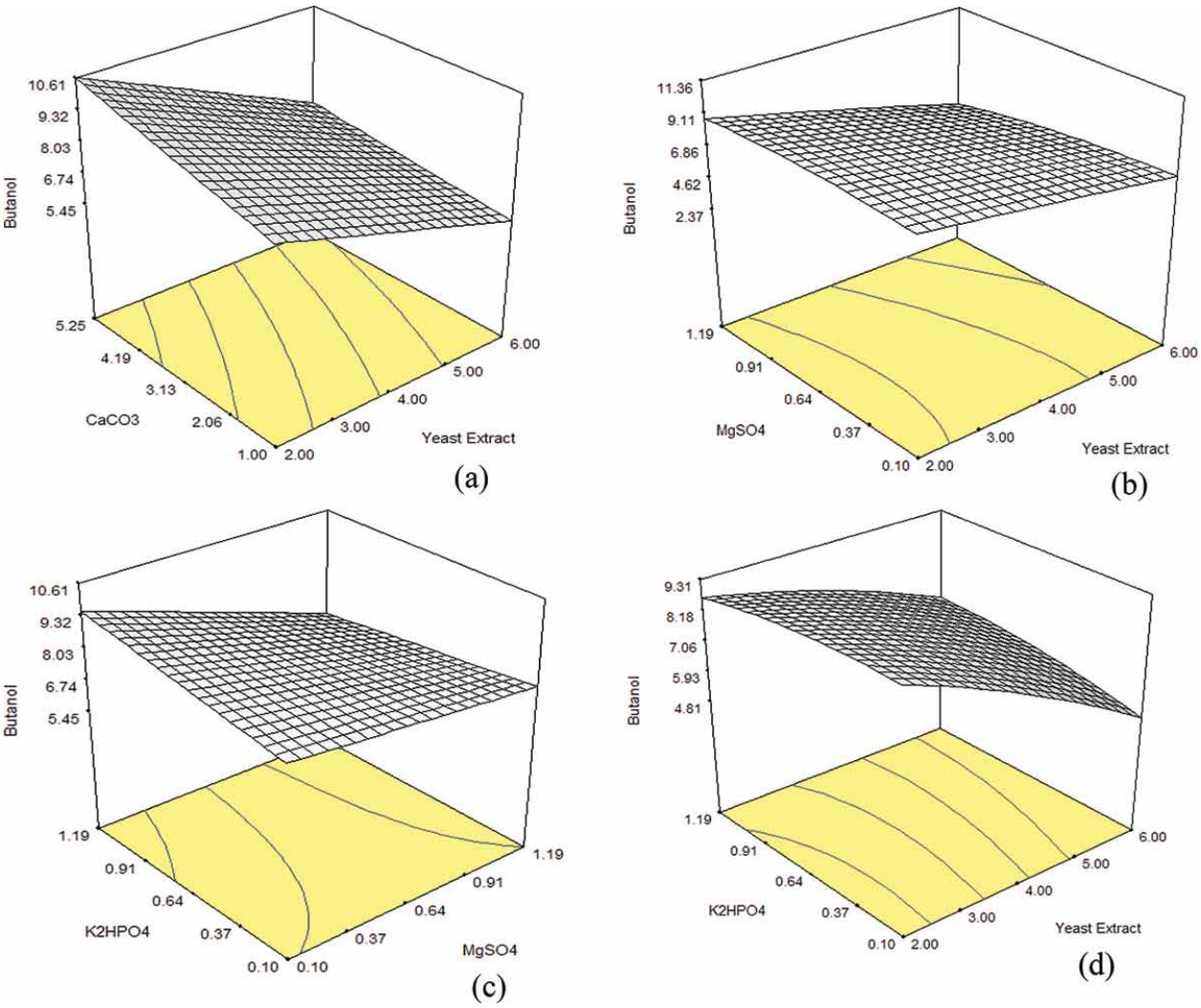

Table 1 Steepest ascent design for biobutanol production

Run No.	Variable (g/L)					Residual glucose (g/L)	Solvent (g/L)				Acids (g/L)	
	Yeast extract	$CaCO_3$	$MgSO_4$	K_2HPO_4	Peptone		Acetone	Butanol	Ethanol	ABE	Butyric	Acetic
1	0.50	0.00	1.00	0.10	0.00	11.27	2.72	7.33	0.86	10.91	1.57	1.57
2	1.00	2.50	0.50	0.50	0.00	1.03	3.10	9.37	1.01	13.49	1.14	1.53
3	2.00	5.00	0.10	1.00	0.00	1.94	2.61	9.18	0.90	12.70	0.03	0.99
4	3.00	0.00	0.10	0.50	0.00	3.12	3.57	8.57	0.93	13.07	1.53	1.70
5	4.00	2.50	0.50	1.00	0.00	2.12	3.41	10.33	1.21	14.94	0.28	0.88

6	4.00	5.00	0.50	1.00	0.00	1.97	3.12	10.51	1.12	14.74	0.30	0.95
7	5.00	5.00	1.00	0.10	0.00	1.88	3.15	9.46	0.87	13.49	0.65	1.08
8	2.50	2.50	0.10	0.10	0.00	1.97	3.46	9.82	0.88	14.16	1.55	1.82
9	2.50	2.50	0.10	0.10	7.00	2.00	3.37	9.31	1.01	13.69	0.37	0.98

Table 2 Experimental design matrix using RSM with CCD and experimental and predicted biobutanol production

Run No.	Variables (g/L)				Response (g/L)	
	Yeast Extract	$CaCO_3$	$MgSO_4$	K_2HPO_4	Experimental	Predicted
1	2.00	1.00	0.10	0.10	8.79	8.88
2	6.00	1.00	0.10	0.10	7.29	6.54
3	2.00	5.25	0.10	0.10	10.42	10.50
4	6.00	5.25	0.10	0.10	6.60	7.00
5	2.00	1.00	1.19	0.10	9.32	9.84
6	6.00	1.00	1.19	0.10	4.01	4.41
7	2.00	5.25	1.19	0.10	10.77	10.52
8	6.00	5.25	1.19	0.10	4.73	3.93

9	2.00	1.00	0.10	1.19	7.99	8.66
10	6.00	1.00	0.10	1.19	8.01	8.02
11	2.00	5.25	0.10	1.19	12.55	11.91
12	6.00	5.25	0.10	1.19	10.77	10.11
13	2.00	1.00	1.19	1.19	7.85	7.20
14	6.00	1.00	1.19	1.19	3.68	3.47
15	2.00	5.25	1.19	1.19	8.90	9.51
16	6.00	5.25	1.19	1.19	4.94	4.62
17	0.00	3.13	0.64	0.64	10.64	10.23
18	8.00	3.13	0.64	0.64	2.22	3.00
19	4.00	0.00	0.64	0.64	7.85	7.48

20	4.00	7.38	0.64	0.64	10.02	10.59
21	4.00	3.13	0.00	0.64	8.97	9.41
22	4.00	3.13	1.73	0.64	6.31	6.53
23	4.00	3.13	0.64	0.00	6.46	6.43
24	4.00	3.13	0.64	1.73	6.50	6.90
25	4.00	3.13	0.64	0.64	8.05	7.67
26	4.00	3.13	0.64	0.64	7.62	7.67
27	4.00	3.13	0.64	0.64	8.16	7.67
28	4.00	3.13	0.64	0.64	6.99	7.67
29	4.00	3.13	0.64	0.64	7.18	7.67
30	4.00	3.13	0.64	0.64	8.16	7.67

Table 3 Model fit summary for biobutanol production

Model	Sum of squares	Degree of freedom	Mean square	F value	*P*value Prob > F
Linear	115.72	4	28.93	18.56	< 0.0001
2FI	138.94	10	13.89	16.76	< 0.0001
Quadratic	147.58	14	10.54	22.25	< 0.0001

Table 4 ANOVA analysis for response surface quadratic model for biobutanol production

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	147.58	14	10.54	22.25	< 0.0001
A-Yeast extract	78.43	1	78.43	165.58	< 0.0001
B-$CaCO_3$	10.14	1	10.14	21.40	0.0003
C-$MgSO_4$	25.12	1	25.12	53.04	< 0.0001
D-K_2HPO_4	0.33	1	0.33	0.70	0.4150
A^2	1.98	1	1.98	4.18	0.0589
B^2	2.81	1	2.81	5.93	0.0278
C^2	1.35	1	1.35	2.84	0.1126
D^2	1.79	1	1.79	3.78	0.0708

AB	1.35	1	1.35	2.84	0.1127
AC	9.58	1	9.58	20.23	0.0004
AD	2.88	1	2.88	6.09	0.0261
BC	0.89	1	0.89	1.88	0.1904
BD	2.65	1	2.65	5.60	0.0318
CD	5.86	1	5.86	12.37	0.0031
Residual	7.11	15	0.47		
Lack of Fit	5.77	10	0.58	2.15	0.2055
Pure Error	1.34	5	0.27		
Cor Total	154.68	29			

Std. Dev.	0.69	R^2	0.954
Mean	7.73	Adj R^2	0.911

Adeq. Precision	18.313