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## Optimization of fermentation conditions for the production of 2,3-butanediol by *Klebsiella pneumonia* ZH-1 using response surface methodology

LI Li, Feng HE, Jian-Guo XU and Qing-Ping HU \*

College of Life Science, Shanxi Normal University, Linfen, Shanxi 041000, China

### ABSTRACT

**[Background]** It has been studied that the yield of 2,3-butanediol (2,3-BD) producing strains is low and does not meet the requirements of industrial production of 2,3-BD. **[Objective]** It was important to improve the production of 2,3-BD by *Klebsiella pneumonia* ZH-1 in shaking flask. **[Methods]** The effects of temperature, initial pH and rotating speed on the production of 2,3-BD were studied by single factor test and response surface method. **[Results]** The optimal cultivation conditions stimulating the maximal production of 2,3-BD were as follow: initial pH, 7, temperature, 37 °C and rotating speed, 140 r/min. Under this optimized conditions, the predicted maximal 2,3-BD yield was 21.54 g/L, whereas the yield of 2,3-BD can reach to 22.04 g/L after the application of response surface methodology. **[Conclusion]** Response surface methodology was a promising method for optimization of 2,3-BD production

**Keywords:** *K. pneumonia* ZH-1, response surface methodology, 2,3-butanediol

### \*Correspondence to Author:

HU Qing-Ping: College of Life Science, Shanxi Normal University, Linfen, Shanxi 041000, China, Telephone: +86 13033426163; Fax: +86 357 2051000; E-mail: hqp72@163.com

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## 1. Introduction

The gradual exhaustion of natural resources has led to the sustainable development of renewable resources, and the interest in 2,3-butanediol (2,3-BD) has increased due to its wide application in the fields of fuels, chemicals, food industry and so on<sup>[1]</sup>. 2,3-BD is a chiral compound with a high boiling point and a low freezing point, which is a colorless and odorless liquid at room temperature<sup>[2]</sup>. As an important starting material, 2,3-BD can be used to produce valuable derivatives such as methyl ethyl ketone and 1,3-butadiene<sup>[3]</sup>. 2,3-BD has been shown to have the potential for the manufacture of printing inks, fragrances, fumigants, wetting and softening agents, explosives and plasticizers, and as a drug carrier<sup>[2]</sup>.

2,3-BD are produced by a mixed acid fermentation route. Acetylcholine (AC) is the main by-product in the fermentation process. Many bacterial species such as *Klebsiella pneumoniae*<sup>[4]</sup>, *Klebsiella oxytoca*<sup>[5]</sup>, *Enterobacter cloacae*<sup>[6]</sup>, *Enterobacter aerogenes*<sup>[7]</sup>, and *Bacillus polymyx*<sup>[8]</sup> can secrete 2,3-BD. Among all these strains, *K. pneumoniae* is one of the best organisms that show the potential for industrial 2,3-BD production. Yu and Saddler<sup>[9]</sup> obtained a diol concentration of 113 g/L by using a supplemental batch operation with *K. pneumoniae*, but the diol production was relatively low (0.94 g/L·h). Although 2,3-BD production has improved, but the concentration and productivity is not enough for economic industrial production. Therefore, it is essential to further improve 2,3-BD production by selecting high-yield strains or system optimization of fermentation conditions.

Response surface methodology, an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization. This method had been successfully applied in the optimization medium compositions<sup>[10]</sup>, conditions of enzymatic hydrolysis<sup>[11]</sup>, and fermentation processes<sup>[12]</sup>. It can give information about the interaction between variables, provide information necessary for design and process optimization, and give multiple responses at the same time.

The aim of this work was apply statistical methods to enhance the production of 2,3-BD by optimizing the fermentation medium conditions of

the *K. pneumoniae* ZH-1. A central composite design (CCD) was used for the fermentation condition optimization to improve the yield of 2,3-BD here<sup>[13]</sup>. This will provide theoretical basis and guidance to reduce costs and increase production for industrial production 2,3-BD.

## 2. Materials and methods

### 2.1 Microorganism

*K. pneumoniae* ZH-1 was originally isolated and identified from Fenhe River (in Shanxi Province, China). After being grown on the solidified of Beef extract-peptone medium containing (per liter): 3.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, 15.0 g bacto-agar and 1000 ml tap water, the strain was stored in a refrigerator at 4 °C.

### 2.2 Preparation of medium

Both inoculum and fermentation medium contained (g/L), glucose, 60; yeast extract, 10;  $\text{KH}_2\text{PO}_4$ , 4;  $\text{K}_2\text{HPO}_4$ , 24;  $(\text{NH}_4)_2\text{SO}_4$ , 2; citrate sodium, 1;  $\text{CuSO}_4$ , 0.04; EDTA, 0.05 distilled water 1 L. The medium was autoclaved for 20 min at 121°C after the pH of the medium was adjusted to 7<sup>[14]</sup>.

### 2.3 Fermentation

Seed cultures were prepared by inoculating cells grown on a beef extract-peptone agar slant into a 250-ml flask that contained 100 ml of the inoculums medium and subsequently incubated at 37 °C for 18 h with shaking at 120 r/min. About 5 milliliters of the seed culture were transferred into the 250-ml flask containing 100 ml of the fermentation media. The culture was shaken at 37 °C and with 140 r/min for 24 h.

### 2.4 Single factor experiments

The purpose of this study was to choose the temperature, initial pH and rotating speed for optimal the production of 2,3-BD, which specifically set to 25 °C, 28 °C, 30 °C, 33 °C, 35 °C, 37 °C, 40°C, 45°C of temperature, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 of initial pH and 0, 40, 80, 100, 120, 140, 160, 180, 200, 220 r/min of rotating speed.

### 2.5 Analytical methods

The optical density (OD) of the culture was assayed using a vis spectrophotometer (722S Jinghua, China) at 600 nm with appropriate dilution.

The pH of the culture medium was recorded using a digital pH meter.

Determination of the products was carried out on an Agilent gas chromatograph (GC) 7820. The GC was equipped with a flame ionization detector and a 30 m capillary column operated with N<sub>2</sub> as carrier gas. The temperatures for the GC were as follows: injector temperature, 250 °C; detector, 250 °C; initial oven temperature, 50 °C for 1 min, followed by 15 °C·min<sup>-1</sup> ramp to 180 °C for a final 10 min hold<sup>[15]</sup>. The sample was firstly extracted by ethyl acetate, and then injected into the gas chromatograph. The concentration of the products was determined by calibration curves<sup>[16]</sup>.

## 2.6 Experimental design

To find the optimal cultivation conditions for 2,3-BD production in batch cultures, the key factors affecting the 2,3-BD production must be determined. Based on the results of our preliminary experiments, the major factors were optimized using response surface methodology (RSM) design (Design-Expert 8.0.6). Table 1 shows the ranges of variables of temperature, initial pH and rotating speed for RSM<sup>[17]</sup>.

## 3. Results and discussion

### 3.1 Effects of temperature for *K. pneumoniae* ZH-1

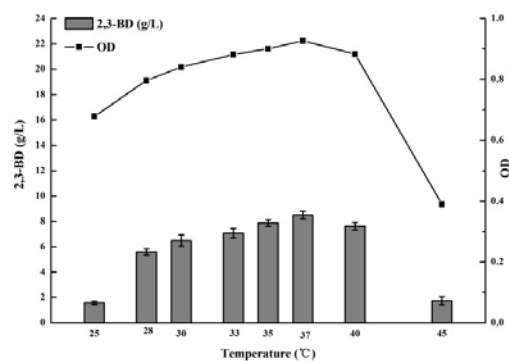


Fig 1: Effect of temperature on the production of 2,3-BD and OD

The results of the production of 2,3-BD and the cell concentration are given in Fig 4, which showed that under the temperature of 25-37 °C fermentating for 24 h, cell concentration and the production of 2,3-BD increased with the temperature. The yield of 2,3-BD reached a maximum of 8.5 g/L when the temperature reached 37 °C. In short, the temperature is too high or too

low will make 2,3-BD production decreased, and the bacterial concentration decreased<sup>[18]</sup>. Therefore, the optimum temperature is 37 °C for the production of 2,3-BD by *K. pneumoniae* ZH-1<sup>[19]</sup>.

### 3.2 Effects of pH for *K. pneumoniae* ZH-1

pH is closely related to the life activities of microorganisms. pH affect the absorption of nutrients by affecting the permeability of the cytoplasmic membrane, the stability of membrane structure and material solubility or ionization. But pH also affect the activity of various enzymes in metabolic reactions<sup>[20]</sup>. Thus, the yield of 2,3-BD is different with the change of pH. As showed in Fig 2, the changes of pH have a significant impact on cell growth and the production of 2,3-BD. Under the pH of 4-7 fermentating for 24 h, the cell concentration and the production of 2,3-BD increased with the pH. The yield of 2,3-BD reaches the maximum of 12.84 g/L when the pH is 7. The yield of 2,3-BD decreases with increasing pH when pH exceeds 7. Thus, the optimum pH for producing 2,3-BD is 7.

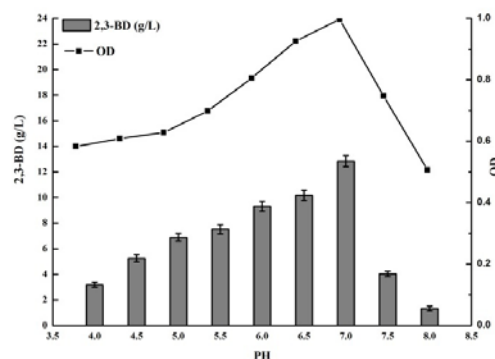


Fig 2: Effect of pH on the production of 2,3-BD and OD

### 3.3 Effects of rotating speed for *K. pneumoniae* ZH-1

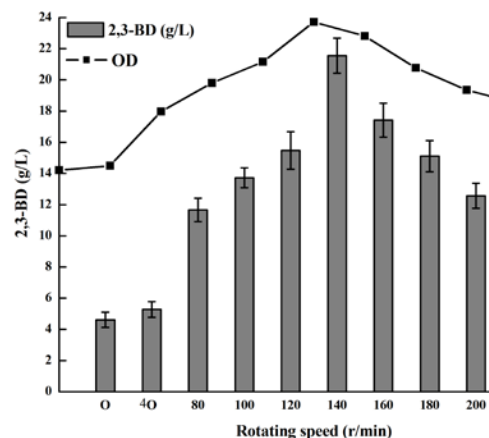


Fig 3: Effect of rotating speed on the production of 2,3-BD and OD

2,3-BD is a typical anaerobic metabolite, but some researchers found that the amount of oxygen can increase the fermentation efficiency and increase the yield of 2,3-BD<sup>[21]</sup>. In the shake bottle conditions, the impact of dissolved oxygen is an important factor in the rotating speed. Don't change the other conditions, the rotating speed of 0 r/min as a control, the rotating speed were set to 40 r/min, 80 r/min, 100 r/min, 120 r/min, 140 r/min, 160 r/min, 180 r/min, 200 r/min and 220 r/min. The effect of the rotating speed on the fermentation of 2,3-BD is showed in Fig 3. The production of 2,3-BD reaches the maximum of 21.55 g/L when the rotating speed is 140 r/min<sup>[22]</sup>.

### 3.4 Optimization by response surface methodology

A total of 15 experiments with combinations of temperature, initial pH and rotating speed were conducted. A central composite design with 3 levels for all the 3 factors: temperature (A), initial pH (B) and rotating speed (C) were used for this purpose. The range of the variables is given in Table 1. The experimental design and the results obtained from experiments are shown in Table 2. The results of this experiments were fitted with a second order polynomial equation<sup>[23]</sup>. The values of regression coefficients were calculated, and the fitted equation (in terms of coded value) for predicting 2,3-BD production (Y) was as given below regardless of the significance of the coefficients:

$$Y=22.54+0.82A+0.49B+0.41C+0.44AB+1.5AC+0.18BC-1.74A^2-3.37B^2-3.73C^2$$

Table 1: Values of coded levels used for the experimental design

Factors	Symbols	Actual levels of coded factors		
		-1	0	1
temperature	A	34	37	40
initial pH	B	6.5	7	7.5
rotating speed	C	120	140	160

Table 2: Central composite design for the experimental design and results

Run numbers	A	B	C	2,3-BD (g.L <sup>-1</sup> )
	tempera- ture(°C)	initial pH	rotating speed	
1	0	0	0	21.96
2	-1	-1	-1	13.39
3	1	1	-1	12.65
4	1	-1	1	14.61
5	-1	1	1	11.32
6	-1.41	0	0	16.61
7	0	0	1.41	14.36
8	0	1.41	0	15.20
9	0	0	0	21.56
10	0	-1.41	0	13.82
11	0	0	0	22.04
12	0	0	0	21.14
13	0	0	0	21.57
14	0	0	-1.41	13.20
15	1.41	0	0	18.92

The statistical significance of the regression model was checked by F-test, and the analysis of variance for the response surface quadratic model is shown in Table 3. The model was highly significant, as manifested by the F-value and the probability value [ (P>F) = 0.0001 ]. The goodness of fit was manifested by the determination coefficient (R<sup>2</sup>). In this case, the R<sup>2</sup> value of 99.33% indicated that the response model can explain 99.33% of the total variations. In general, a regression model having an R<sup>2</sup> value higher than 0.9 is considered to have a very high correlation. The value of the adjusted determination coefficient (R<sup>2</sup><sub>Adj</sub> = 98.13%) was also high enough to indicate the significance of the model.

The optimum of location, obtained by differentiation of the quadratic model, for achieving maximal 2,3-BD production was A = 37 °C, B = 7 and C = 140 r/min. The predicted optimal 2,3-BD production corresponding to these values was 21.54 g/L. To confirm the goodness of the model for predicting maximal 2,3-BD production. Additional experiments in triplicates using these optimized fermentation condition carried out. These triplicate experiments yielded an average maximum 2,3-BD production of 22.04 g/L. The good agreement between the predicted and experimental values confirms the validity of the model and the existence of optimum point.

The 3D response surfaces plots and 2D contour

plots were employed to determine the interaction of the fermentation conditions and the optimum levels that have the most significant effect on 2,3-BD production<sup>[24]</sup>. The response surfaces plots and their respective 2D contours have been depicted in Fig 1, Fig 2 and Fig 3. It is cleared from Fig 1 that the minimum response of 2,3-BD production (15.54 g/L) occurred when temperature was at its lowest level. 2,3-BD production increased considerably as temperature increased, indicating that temperature for 2,3-BD production has a significant effect on the responses. As temperature increased, the responses were maximal nearly at the middle of initial pH. The response was also varied at different levels of initial pH along the axis, suggesting that there is a considerable interaction between temperature and initial pH (Fig 1). In other reports, optimal conditions for 2,3-BD production were obtained at an initial pH of 5, 6.0<sup>[14]</sup>, 6.5<sup>[25]</sup> and 7.5. The different optimal initial pH values reported in the literature may be due to the different strains. Fig 2 demonstrates the effects of rotating speed and temperature on 2,3-BD production. The 2,3-BD production was affected by the rotating speed and temperature, and also there is a considerable interaction between them for 2,3-BD production. Similarly Fig 3 shows the effects of rotating speed and initial pH on the 2,3-BD production. Response surfaces optimization supported 30 g/L production of 2,3-BD by *K. pneumonia* ZH-1.

Table 3: Analysis of variance for response surface quadratic model obtained from experimental results.

Source	Sum of squares	df	Mean squares	F-value	Probability > F
Model	215.43	9	23.94	82.43	0.0001
A	2.67	1	2.67	9.19	0.0290
B	0.95	1	0.95	3.28	0.1299
C	0.67	1	0.67	2.32	0.1885
A2	23.33	1	23.33	80.35	0.0001
B2	87.44	1	87.44	301.09	0.0001
C2	107.42	1	107.42	369.92	0.0001
AB	0.38	1	0.38	1.32	0.3027
AC	4.47	1	4.47	15.4	0.0111
BC	0.064	1	0.064	0.22	0.6579
Residual	1.45	5	0.29		
Lack of fit	0.93	1	0.93	7.11	0.056
Pure error	0.52	4	0.13		
Core total	216.88	14			

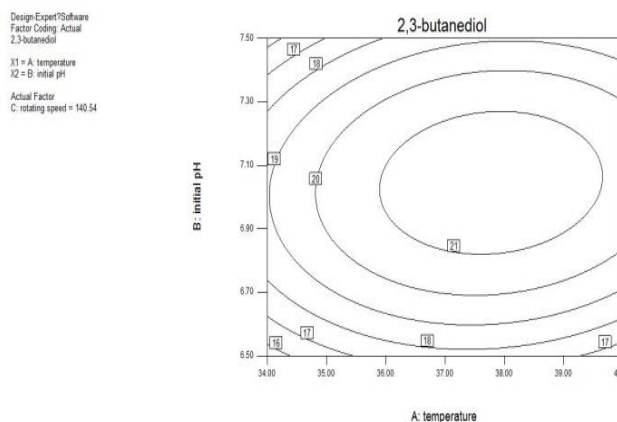
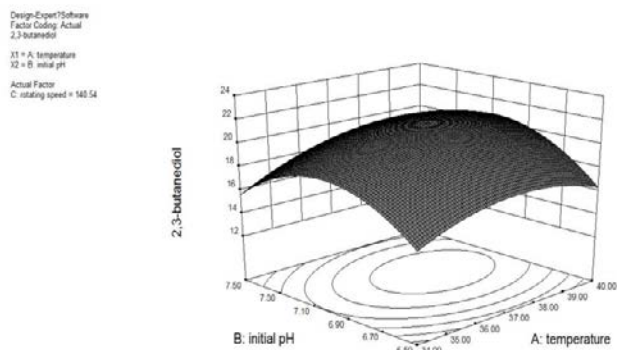


Fig 1: Response surface and corresponding contour for 2,3-BD production by *K.pneumonia* ZH-1. The interaction between initial pH and temperature

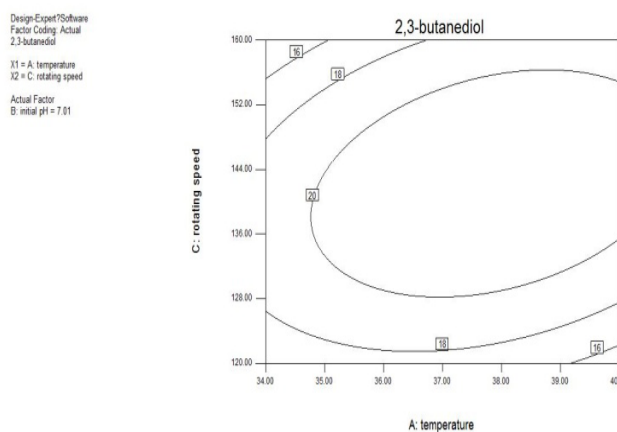
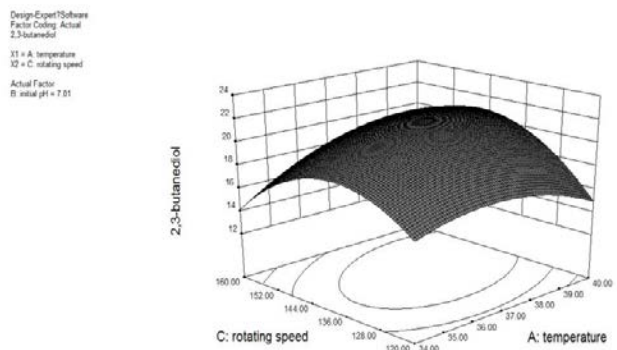


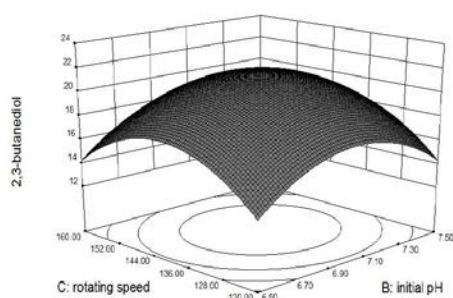
Fig 2: Response surface and corresponding contour for 2,3-BD production by *K.pneumonia* ZH-1. The interaction between rotating speed and temperature.

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Factor Coding Actual  
2,3-butanediol  
X1 = B: initial pH  
X2 = C: rotating speed  
Actual Factor  
A: temperature = 37.08



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X1 = B: initial pH  
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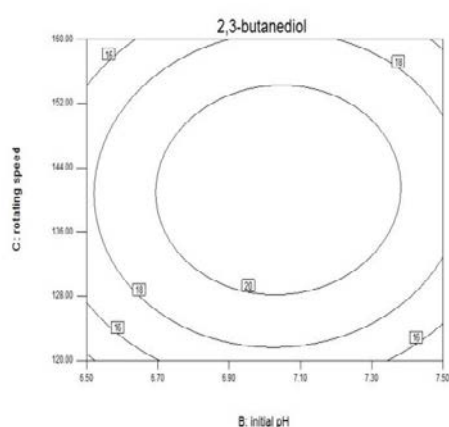


Fig 3: Response surface and corresponding contour for 2,3-BD production by *K.pneumonia* ZH-1. The interaction between rotating speed and initial pH.

## 4. Conclusions

Statistical optimization of fermentation medium could overcome the limitations of classical empirical methods. It was proved to be a powerful tool for the optimization of the 2,3-BD production by *K. pneumonia* ZH-1. Response surfaces methodology was proposed to study the combined effects of culture medium components. The existence of interaction between the independent variables with the responses was observed. The optimum fermentation conditions are as follows: temperature, 37 °C; initial pH, 7 and rotating speed, 140 r/min. After the optimization, the 2,3-BD yield increased to 22.04 g/L over a fermentation period of 24 h when using the optimized fermentation conditions. It was shown that statistical experimental design offered an effect and feasible approach for 2,3-BD fermentation medium optimization.

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