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RESEARCH ARTICLE

ORTHOGONAL TEST DESIGN TO OPTIMIZE THE PRODUCTION OF 2,3-BUTANEDIOL ON FERMENTATION MEDIUM BY KLEBSIELLA PNEUMONIA ZH-1

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ABSTRACT

There has been a growing interest in the fermentative production of 2,3-butanediol (2,3-BD) because of its wide applications in the chemical, food, pharmaceutical, and fuel industries. In this study, the increase in 2,3-BD production was achieved by optimizing the fermentation medium. The strains of Klebsiella pneumonia ZH-1, was studied by single factor and orthogonal test to select the best fermentation medium. Carbon source, nitrogen source, mineral salt and metal ion have an important effect on the cell growth of K. pneumonia ZH-1 and the production of 2,3-BD. The optimal fermentation medium were determined as follows (g/L): glucose 60; yeast extract 10; KH2PO4 4; K2HPO4 24; (NH4)2SO4 2; citrate sodium 1; $CuSO_4$ 0.04; EDTA 0.05. Among them, the biggest influence is yeast extract on this test result. Under the optimal conditions, the output of 2,3-BD can reach 13.5 g/L, which provide theoretical basis for industrialized production of 2,3-BD and the instruction.

INTRODUCTION

With the depletion of fossil resources, fermentative production of 2,3-butanediol (2,3-BD) from microbial fermentation has received increasing interest because of its wide substrate spectrum, high efficiency, and cultural adaptability. 2,3-BD is one of the important chemical intermediate, which can be used in many fields such as food, chemistry, pharmaceutical, fue. *Klebsiella pneumonia* ZH-1 is considered to be one of the most industrialized potential and is one of the 2,3-BD production strains with its broad substrate utilization ability, high production efficiency. 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 (Garg and Jain, 1995). As an important starting material, 2,3-BD can be used to produce valuable derivatives such as methyl ethyl ketone and 1,3-butadiene (Syu, 2001).

In recent years, biological production of biodiesel, bioethanol, 1,3-propanediol (Kumar *et al*., 2016) and other chemical products have been widely attention, the corresponding research and industrialization development both at home and abroad are relatively fast (Hong *et al*., 2015). However, 2,3-BD as a kind of important chemical raw materials and liquid fuels (Zhang, 2008), has been not realized industrialized mass production because of its special structure, the chemical synthesis of 2,3-BD cost is very high.

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Biotransformation production 2,3-BD is a renewable resource as raw material, through the microbial metabolism of monosaccharides into the target product (Jansen and Tsao, 1983). Compared with the chemical method, biotransformation method is consistent with the requirements of environmental friendly, and can reduce the human dependence on the increasingly depleted oil resources, which has a good application prospects. The strain of *Enterobacter aerogenes* produce 2,3-BD by using of cell cycle technology, which made the production of 2,3-BD obtain 110 g/L (Zeng *et al*., 1998). *K. pneumoniae* was fermented by batch feeding to obtain 2,3-BD and 3-hydroxy -2-butanone for a total of 113 g/L (Yu and Sadller, 1983).

Domestic, Shandong University made the 2,3-BD production reached 105.5 g/L by batch feeding method (Qin *et al*., 2006). Using *K. pneumonia* DSM 2026 as the strain (Sun, 1986), the optimum conditions for the production of 2,3-BD by biotransformation were determined by batch fermentation experiment: initial substrate concentration 8%, micro-oxygen fermentation, 0.04 vvm, pH 5.5 (Cheng *et al*., 2013). Under the conditions of batch fermentation, the target product concentration was 42.60 g/L, the production intensity was 0.76 g/(L.h), and the mass conversion rate of glucose was 23%. Based on this, the dynamic behavior of *K. pneumonia* DSM 2026 in continuous fermentation under microaerobic conditions was investigated (Wong *et al*., 2014). The results showed that the concentration of substrate could not induce long-term oscillation, but the concentration of substrate of the short-period oscillation of CO_2 occurs without disturbing (Gao *et al*., 2016), and this oscillation occurs only when the dilution rate is in the range of 0.20-0.25 h^{-1}. The oscillation period is

9.3-15.0 min. In summary, the biological production of 2,3-BD is the most ideal way. However, the reported yield of the strain is low, the product purity is not high, cannot meet the requirements of industrial production, and the fermentation conditions of different strains are different. Therefore, we selected a strain with high production of 2,3-BD, and optimized its fermentation medium. The single factor and orthogonal test were used to study the effect of *K. pneumonia* ZH-1 to the best fermentation medium to increase the production of 2,3-BD.

MATERIALS AND METHODS

Microorganism and medium

K. pneumonia 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, the strain was stored in a refrigerator at 4 °C (Zhao, 2008). Seed medium (g/L): glucose, 20; peptone 10; yeast extract 5; NaCl 10; pH 7; 0.1 MPa sterilized for 20 min. Without optimization of 2,3-BD basic fermentation medium (g/L): glucose 40; yeast extract 5; KH_2PO_4 6; K_2HPO_4 14; $(NH_4)_2SO_4$ 2; Sodium citrate 1; $MgSO_4 \cdot 7H_2O$ 0.4; EDTA 0.05; pH 7.0; 0.1 MPa sterilized for 20 min. Seed cells for fermentation were prepared in a 250 mL flask containing 100 mL preculture medium. Flask cultures were incubated at 37 ^oC for 18h and subsequently inoculated into the fermentation medium at a concentration of 5% (v/v). The rotating speed was adjusted to 120 rpm. The media was altered to determine the optimal formulation for subsequent experiments, the pH value was maintained at 7.0 by adding NaOH or HCl.

Extraction

The cells of *K. pneumonia* ZH-1 grown in the fermentation medium for 24 hours were centrifuged at 6000 r/min for 10 min, and 2,3-BD in the supernatant was extracted in a 1: 1 by volume basis of ethyl acetate, the evaporator spins away the other material with a lower boiling point than 2,3-BD. The remaining material through the aperture of 0.22 um organic membrane filter into the chromatographic flask, through the gas chromatograph can be measured 2,3-BD content.

Analytical methods

2,3-BD were analyzed by injecting 1 µl of reaction mixture into a gas chromatograph equipped (Aglient GC7820) with a capillary column (ON-Wax, 30 m×0.32 mm×0.5 µm). The circumstance under the operation were as follow: nitrogen was used as the carrier gas; the injector temperature and the detector temperature were both 250°C; and the column oven temperature was maintained at 40°C for 10 min, then raised to 180°C at a rate of 15°C/min (Ma *et al*., 2009). Standard curve was drawn according to the peak area of 2,3-BD standard substance. The concentration of 2,3-BD standard substance were 2.5 g/L, 5 g/L, 10 g/L, 20 g/L, 40 g/L, respectively. Then using the standard curve (y=3E+6x+1E+6, R^2=0.998) to calculate the content of 2,3-BD by strain ZH-1. Growth of the bacteria was monitored by measuring the optical density at 600nm (OD_{600}) of the culture broth using a spectrophotometer (Lee *et al*., 2013).

Single Factor experiments for studying the 2,3-BD production

Single-factor experiments were conducted for studying the growth of *K. pneumonia* ZH-1 and the yield of 2,3-BD under different media. In carbon source experiments, glucose, sucrose, maltose, lactose, glycerol and soluble starch were respectively used as the sole carbon source. The culture medium without carbon source was used as a control group. After setting the optimum carbon source, set the different concentration gradient, select the best carbon source concentration. After determining the optimum carbon source and its optimum concentration, the optimum nitrogen source are set in the same way as above. Nitrogen source were respectively KNO_3, urea, beef extract, yeast extract, peptone, $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$. We regard K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$ and sodium citrate as inorganic salts to study their respective optimum concentration. The concentration gradients of K_2HPO_4 were 0-28 g/L. The concentration gradients of KH_2PO_4 were 0-14 g/L. The concentration gradients of $(NH_4)_2SO_4$ and sodium citrate were both 0-5 g/L. After determining the optimum concentration of inorganic salts, Zn^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+} were studied as metal ion to select the optimum metal ion and its optimum concentration. The concentration gradient of Mg^{2+} Ca^{2+} were 0.1-0.7 g/L and the concentration gradient of Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+} were 0.01-0.07 g/L (Zhao, 2008). All of the above fermentation mediums were loaded into a 250 ml shake flask at a volume of 100 ml, inoculation size of 5% (v/v), the initial pH 7.0 of all fermentation experiments was carried out at a culture temperature of 37 °C and an oscillating speed of 120 rpm for 24h.

Orthogonal experiment design

According to the results of the single factor experiment, orthogonal array ($L9(3^4)$) with four factors at three levels was chosen to seek the optimum conditions for the maximal yield of 2,3-BD. Factors and its levels of orthogonal design were shown in Table 1 and the results of orthogonal test are shown in Table 2 and Table 3. Each of the 9 experiments was performed in triplicate (Gao *et al*., 2016).

Table 1 Orthogonal factors and levels

	Factors			
Level	**A(glucose/g.L^{-1})**	**B(yeast extract/g.L^{-1})**	**C(K_2HPO_4 /g.L^{-1})**	**D($CuSO_4$/ g.L^{-1})**
1	40	0	20	0.04
2	60	5	24	0.07
3	80	10	28	0.1

Statistical analysis

The values are expressed as means ± standard deviation. Statistical analysis was done by SPSS statistical software (SPSS 16.0 version for windows, SPSS, Inc., Chicago, IL, USA). Analysis of variance (ANOVA) is one of the most important statistical tools, which is used to uncover the main factor and interaction effects of variables (Wang *et al*., 2015). It is also used to identify the procedure parameters that are statistically significant. The criterion for statistical significance was $p < 0.05$, and extreme significance was $p < 0.01$.

RESULTS AND DISCUSSION

Optimum of carbon source for K. pneumoniae ZH-1

Carbon source is an important factor affecting the production of 2,3-BD and cell growth, so optimum certain carbon source

at a concentration range is beneficial to the bacterial growth and the production of 2,3-BD (Ramachandran and Goma, 1988). The results in Fig 1 illustrated that the production of 2,3-BD were significantly different among the tested carbon sources ($p < 0.05$). When the strain was fermented with glucose as the carbon source, the cell growth was better and the yield of 2,3-BD was higher.

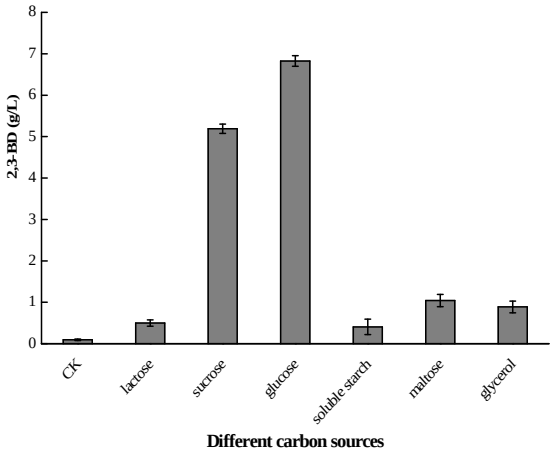

Fig 1 The production of 2,3-BD under different carbon source.

This is determined by the enzyme's own enzyme system. Therefore, for *K. pneumonia* ZH-1, glucose was selected as the optimal carbon source in subsequent experiments, and the optimum concentration of glucose was determined to be 60 g/L (Kim *et al*., 2015).

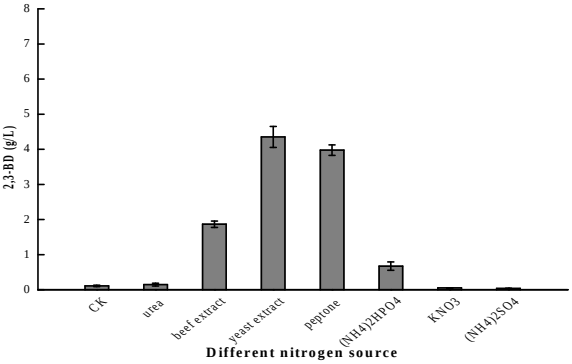

Fig 2 The production of 2,3-BD under different nitrogen source.

Nitrogen is an element that requires microbial cells to be second only to carbon, and is the microbial cell needs only after the carbon element (Campbell *et al*., 1991). Nitrogen sources, the critical media component, were optimized to enhance the production of 2,3-BD by fermental cultivation of *K. pneumonia* ZH-1.The results in Fig 2 revealed that the changes of nitrogen source have a significant impact on cell growth and product synthesis. When the strain was fermented with yeast extract as the nitrogen source, the cell growth was better, because yeast extract can provide a wide range of amino acids, peptides, vitamins, inorganic salts, and carbon in fermentation medium. Subsequently, we also made different concentrations of yeast extract on the effect of the production of 2,3-BD, the best concentration of yeast extract was 5 g/L.

Optimum of inorganic salts for K. pneumoniae ZH-1

The role of inorganic salts in microbial fermentation is to form bacterial cell components (Guillard *et al*., 2003). Therefore, it is necessary to know the optimum amount of inorganic salt in the production by experiment to stabilize or increase the yield.

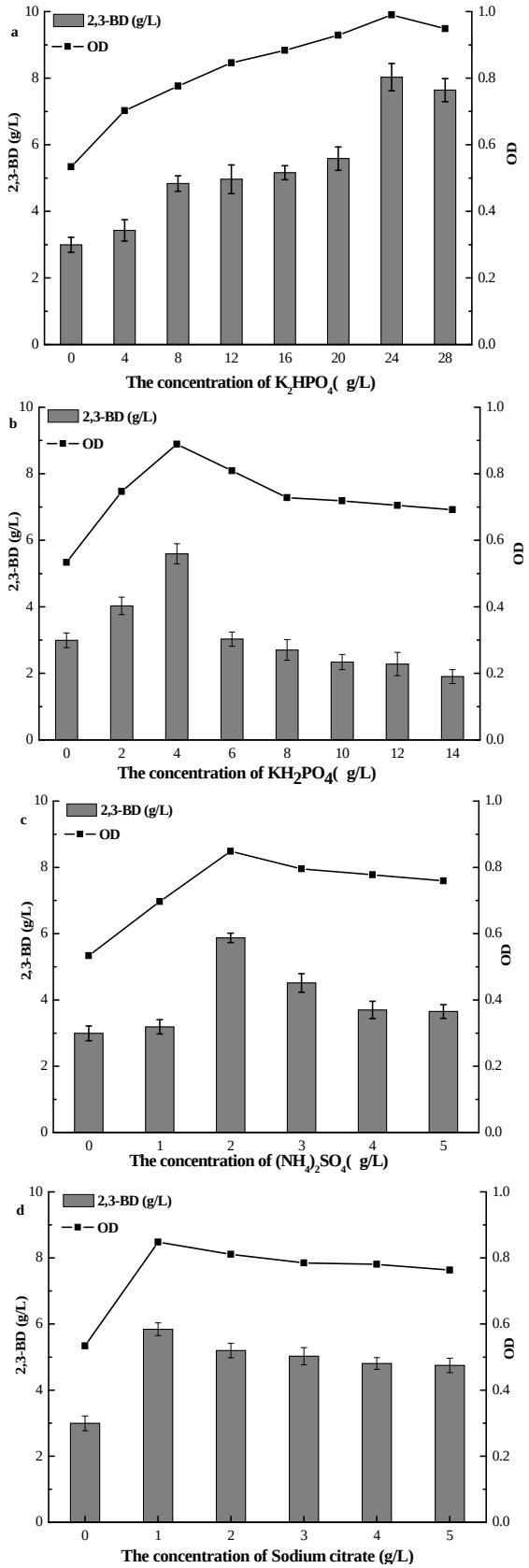

Fig 3 The production of 2,3-BD under different inorganic salts: K_2HPO_4 (a), KH_2PO_4 (b),$(NH_4)_2SO_4$ (c), and Sodium citrate (d)

As showed in Fig 3, the effect of different inorganic salts on the production of 2,3-BD was different. K_2HPO_4 had a greater effect on the amount of 2,3-BD produced by *K. pneumonia*

ZH-1 than other inorganic salts. With the increase of K_2HPO_4 concentration in the fermentation medium, the yield of 2,3-BD gradually increased. When the appropriate concentration (24 g/L) of K_2HPO_4 was added to the fermentation medium, the amount of 2,3-BD (8.03 g/L) was significantly higher than that without K_2HPO_4 (2.99 g/L), making the yield of 2,3-BD increased by about 1.7 times. At the same time, the amount of bacteria reached its maximum. Therefore, the optimum concentrations of K_2HPO_4 , KH_2PO_4, $(NH_4)_2SO_4$ and Sodium citrate were respectively 24 g/L, 4 g/L, 2 g/L, 1 g/L.

Optimum of metal ion for K. pneumoniae ZH-1

inhibitory effect on the yield of 2,3-BD in the selected concentration range. Therefore, the optimum concentration of Cu^{2+} in the fermentation medium is 0.07 g/L, the yield of 2,3-BD can reach 12.34 g/L.

Orthogonal test for the production of 2,3-BD of the K. pneumonia ZH-1

The orthogonal experiment results were tabulated in Table 3. Variance analysis (Table 4) indicated that larger range value indicated that the composition of the culture medium makes a greater impact on the production of 2,3-BD by *K. pneumonia* ZH-1.

Table 2 Effect of concentration of metal ion on the yield of 2,3-BD

metal element	Concentration (g/L)	2,3-BD (g/L)	metal element	Concentration (g/L)	2,3-BD (g/L)
	0.01	8.27±0.34		0.01	7.50±0.23
Zn^{2+}	0.04	8.25±0.41	Ni^{2+}	0.04	9.90±0.46
	0.07	7.09±0.36		0.07	10.30±0.24
	0.1	5.99±0.30		0.01	8.67±0.31
Ca^{2+}	0.4	7.41±0.25	Cu^{2+}	0.04	9.47±0.35
	0.7	9.26±0.42		0.07	12.34±0.40
	0.01	8.15±0.39		0.01	8.97±0.36
Fe^{2+}	0.04	9.03±0.47	Mn^{2+}	0.04	9.18±0.47
	0.07	11.36±0.30		0.07	9.35±0.33
	0.1	8.14±0.38		0.01	9.86±0.44
Mg^{2+}	0.4	8.95±0.40	Co^{2+}	0.04	9.22±0.47
	0.7	8.79±0.26		0.07	7.17±0.31
CK		7.86±0.35			

Table3 Results of orthogonal experiment

	Factor A(glucose/g.L^{-1})	Factor B(yeast extract/g.L^{-1})	Factor C(K_2HPO_4/g.L^{-1})	Factor D($CuSO_4$/g.L^{-1})	2,3-BD (g.L^{-1})
1	40(A_1)	0(B_1)	20(C_1)	0.04(D_1)	4.64
2	40(A_1)	5(B_2)	24 (C_2)	0.07 (D_2)	11.07
3	40(A_1)	10(B_3)	28 (C_3)	0.10(D_3)	11.65
4	60(A_2)	0(B_1)	24(C_2)	0.10(D_3)	5.65
5	60(A_2)	5(B_2)	28(C_3)	0.04(D_1)	11.31
6	60(A_2)	10(B_3)	20(C_1)	0.07(D_2)	10.48
7	80(A_3)	0(B_1)	28(C_3)	0.07(D_2)	1.16
8	80(A_3)	5(B_2)	20(C_1)	0.10(D_3)	9.22
9	80(A_3)	10(B_3)	24(C_2)	0.04(D_1)	11.51
K1	9.12	3.82	8.11	9.15	
K2	9.15	10.53	9.41	7.57	
K3	7.29	11.21	8.04	8.84	
R	1.85	7.39	1.37	1.58	
The optimal levels		$A_2B_3C_2D_1$			
Major and minor factors		B > A > D > C			

Table 4 ANOVA data of orthogonal tests with software SPSS 16.0.

Source	Type Ⅲ Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	391.576[a]	8	48.947	84.648	0
Intercept	2229.806	1	2229.806	3856.203	0
A	23.056	2	11.528	19.937	0
B	342.005	2	171.002	295.73	0
C	12.146	2	6.073	10.503	0.001
D	14.369	2	7.184	12.425	0
Error	10.408	18	0.578		
Total	2631.79	27			
Corrected Total	401.984	26			

In the process of microbial growth and product synthesis also need some trace elements, which are mainly an important part of microbial metabolism in the process of enzyme activity center (Lin, 2011). The results in Table 2 illustrated that Ca^{2+}, Fe^{2+}, Ni^{2+} and Cu^{2+} can promote the production of 2,3-BD. Mg^{2+} and Mn^{2+} can also promote the production of 2,3-BD, but the effect is not obvious, while Zn^{2+} and Co^{2+} has a certain

So the influences of four factors on the production of 2,3-BD can be ranked as follows: B > A > D > C. Therefore, the difference in the concentration of yeast extract influence the production of 2,3-BD the most, followed by the glucose, K_2HPO_4 and $CuSO_4$. And the optimized medium composition was achieved from the orthogonal experiment as follows: the

glucose 60 g/L, the yeast extract 10 g/L, K_2HPO_4 24 g/L and the $CuSO_4$ 0.04g/L (Sun *et al*., 2009).

CONCLUSIONS

The orthogonal experiment results that the optimal fermentation medium for the production of 2,3-BD were: glucose (60 g/L) as carbon source, yeast extract (10 g/L) as nitrogen source, K_2HPO_4 (24 g/L) as inorganic salts and $CuSO_4$ (0.04 g/L) as metal ion. Under the conditions, the *K. pneumonia* ZH-1 exhibited efficient ability with the production of 2,3-BD, which can reach 13.5 g/L. What more, the *K. pneumonia* ZH-1 is one of the most commercially available 2,3-BD producing strains with its broad substrate utilization and high productivity. Due to 2,3-BD as an important chemical raw materials, this study provide theoretical basis and guidance for industrial production 2,3-BD to reduce costs and increase production.

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