



# Optimization of Fermentation Conditions of Glycerol to 1,3-propanediol by an Alkali-resistant *Klebsiella pneumonia* ZH-1 Using Response Surface Methodology (RSM)

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## Authors' contributions

This work was carried out in collaboration between all authors. Author ZFZ managed literature searches, analyses the study, wrote the protocol and carried out laboratory experiments under the supervision of authors JGX and QPH. Author QPH designed the study and performed the statistical analysis. All authors read and approved the final manuscript.

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## ABSTRACT

1,3-propanediol (1,3-PD) is an important chemical material and can be used as monomer to produce polyesters, polyethers and polyurethanes. Its biosynthetic method arouses our more and more interests. In this study, 1,3-PD was produced from crude glycerol through the fermentation of a *Klebsiella* sp. ZH-1 strain isolated from a anaerobic sludge collected in Fenhe river. The fermentation condition of 1,3-propanediol from strain ZH-1 was optimized in 3 aspects of temperature, pH and inoculum by one factor at a time and response surface methodology. Results showed that the optimal fermentation parameters for temperature, pH and inoculum were determined as 36°C, 7.8 and 11%, respectively. Under these conditions, the practical yield of 1,3-PD was 19.93 g-L<sup>-1</sup> and a molar yield (mol<sub>1,3-PD</sub>-mol<sub>Glycerol</sub><sup>-1</sup>) of 1,3-propanediol to glycerol of 0.52. Compared with other strains of producing 1,3-PD by fermentation, the strain *K. pneumoniae* ZH-1 has a higher molar conversion rate. Meanwhile, we believe the strain ZH-1 is an alkaline resistant strain.

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**Keywords:** 1,3-propanediol; *K. pneumoniae*; alkali-resistant; fermentation conditions; RSM.

## 1. INTRODUCTION

1,3-propanediol (1,3-PD) is a promising renewable resource. It has a large range of potential utilizations: 1,3-PD can be used for the synthesis of polyurethanes, as a chain extender, lubricant, solvent and precursors for the chemical and pharmaceutical industries [1]. In recent years, the rapid development of 1,3-propanediol is due to its important and irreplaceable role as a monomer in the synthesis of polyethylene terephthalate (PET) [2,3]. PET was a kind of high quality polyester material, it has much more advantages than polyethylene terephthalate (PET) and polybutylene terephthalate (PBT), such as strong flexibility, anti fouling, easy coloring, low static, excellent resilience, and good biodegradability and recycling [4]. Due to the excellent properties of PET, the industrialization of 1,3-PD has been promoted.

Currently, the method of synthesizing 1,3-PD mainly include chemical synthesis and microbial fermentation. The chemical production of 1,3-PD requires high pressure (1500 PSI) and temperature (90°C) conditions as well as expensive catalysts [5]. Therefore, the microbial conversion of glycerol to 1,3-PD has recently gained a great deal of attention and has been subsequently studied in a large number of papers [6]. Glycerol is the substrate of 1,3-PD fermentation, recently its production has increased due to the development of biodiesel industry, and the price decline of it saves costs for the fermentation process of 1,3-PD and it can speed up the commercialization of microbial approach to 1,3-PD production [7]. In short, microbial fermentation is environmentally friendly and economical.

Numerous species of *Enterobacteriaceae* are able to convert glycerol into 1,3-PD. The most promising ones are *Klebsiella pneumoniae*, *Citrobacter freundii* and *Clostridium butyricum* [8-11]. In all these cases, 1,3-PD was produced under anaerobic conditions in the presence of pure glycerol as the unique carbon source [1]. *Citrobacter freundii* is strictly anaerobic and difficult to cultivate, while *Klebsiella pneumoniae* and *Clostridium butyricum* are facultative anaerobic bacteria and they have a strong glycerol tolerance and high fermentation intensity [6]. Therefore, *Klebsiella pneumoniae* and *Clostridium butyricum* are widely used in the production of 1,3-PD.

However, in all of the wild 1,3-PD producers characterized to date, there is a relatively lower fermentation pH. Silva et al. [12] researched shown the optimum pH range for *Klebsiella pneumoniae* GLC29 was 6.9-7.1. The fermentation of glycerol by *C. butyricum* was regulated under a pH of 7.0 [13]. 1,3-PD was produced by *C. pasteurianum* under a pH of 6.5 [14]. In our previous investigation, we isolated a strain ZH-1 which has the ability of high produce with 1,3-PD under alkaline conditions (pH was 8.0), from Fenhe River in China. It was classified as a member of *K. pneumoniae* after the study of phenotypic, physiological, biochemical and phylogenetic (16S rDNA). The aim of this work is to study the capability of the novel strain ZH-1 in 1,3-PD production from glycerol, and to determine adequate experimental batch conditions such as temperature, pH control strategy, and the inoculum conditions by one factor at a time (OFAT) and response surface methodology (RSM) [6]. This will provide an important step in the development of more successful strategies aiming at exploiting more beneficial microbe resources for 1,3-PD.

## 2. MATERIALS AND METHODS

### 2.1 Media

For enrichment and isolation of cultures the following media was used (per litre): glycerol, 50.0 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 g; K<sub>2</sub>HPO<sub>4</sub>, 3.4 g; KH<sub>2</sub>PO<sub>4</sub>, 1.3 g; MgSO<sub>4</sub>, 0.2 g; CaCl<sub>2</sub>, 2×10<sup>-3</sup> g; FeSO<sub>4</sub>, 2×10<sup>-3</sup> g; yeast extract powder 1 g; citric acid 0.42 g. Trace element solution 0.2% (v/v) was added to the enrichment media (concentration per liter of deionized water): CoCl<sub>2</sub>·6H<sub>2</sub>O 0.2 g; MnCl<sub>2</sub>·4H<sub>2</sub>O 0.1 g; ZnCl<sub>2</sub> 0.07 g; H<sub>3</sub>BO<sub>3</sub> 0.06 g; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.035 g; CuCl<sub>2</sub>·2H<sub>2</sub>O 0.02 g; NiCl<sub>2</sub>·6H<sub>2</sub>O 0.025 g. the initial pH at 7.0 regulated by KOH. 18.0 g agar added while the solid medium was needed [15].

### 2.2 Culture Condition

In this study, the initial glycerol concentration of all experiments was 50 g·L<sup>-1</sup>, and 36 h was cultured in CO<sub>2</sub> incubator (ESCO, America).

### 2.3 Optimization of Fermentation Conditions

OFAT and RSM were chosen to show the statistical significance of the effects of

temperature, inoculum concentration and pH on the production of 1,3-PD by *K. pneumoniae* ZH-1. The RSM experiments were designed by using the Design-Expert 8.0.6. Calculations were done at 95% of confidence level. In order to optimize the incubation conditions and investigate effects of above independent variables on the yield of 1,3-PD, a central-composite rotary design with the variables at three levels was used in the experiments.

## 2.4 Analytical Methods

1,3-PD were analyzed by injecting 0.6 $\mu$ l of reaction mixture into a gas chromatograph equipped(Aglient GC7820) with a capillary column (ON-Wax, 30 m $\times$ 0.32 mm $\times$ 0.5  $\mu$ m). The flow rate of the carrier gas (nitrogen) was 25 ml $\cdot$ min $^{-1}$ . The column temperature was raised to 180 $^{\circ}$ C at 15 $^{\circ}$ C $\cdot$ min $^{-1}$  and maintained for 10 min, while injector and FID detector temperature were both 250 $^{\circ}$ C. Standard curve was drawn according to the peak area of 1,3-PD standard substance. The concentration of 1,3-PD standard substance were 2.5 g $\cdot$ L $^{-1}$ , 5 g $\cdot$ L $^{-1}$ , 10 g $\cdot$ L $^{-1}$ , 20 g $\cdot$ L $^{-1}$ , 40 g $\cdot$ L $^{-1}$ , respectively. Then using the standard curve ( $y=1.5E+6x+134372$ ,  $R^2=0.9993$ ) to calculate the content of 1,3-PD by strain ZH-1.

Residual glycerol was determined by the improved Potassium Permanganate oxidation method according to Wang et al. [16].

Cell growth was monitored at 650 nm (OD 650) on a spectrophotometer (722S Jinghua, China).

## 3. RESULTS AND DISCUSSION

### 3.1 Optimum of Temperature for *K. pneumoniae* ZH-1

In the fermentation process of growth, whether it is solid or liquid fermentation fermentation temperature, inoculum and pH all have a significant impact on its fermentation. In the initial glycerol concentration of 50 g $\cdot$ L $^{-1}$  and fermentation 36 h, we studied the influence of temperature, inoculum concentration and pH on 1,3-PD production from glycerol fermentation by the strain ZH-1. The results (Fig. 1) showed that the temperature of 20-35 $^{\circ}$ C under fermentation for 36 h, cell concentration, 1,3-PD yields and molar conversion rate increased with the temperature. However, when the fermentation temperature exceeded 40 $^{\circ}$ C, 1,3-PD production has been significantly reduced, and as opposed

to residual amounts of glycerol significantly increased. Lower fermentation temperature induced the slower growth of the bacteria, and thus also affect the formation of the 1,3-PD, but higher temperatures inhibit the growth of bacteria and the formation of the 1,3-PD. According to previous reports [17], the optimum temperature for the growth of *K. pneumoniae* and 1,3-PD formation were 30-37 $^{\circ}$ C, our research is consistent with the report.

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

The changes of pH value have a significant impact on cell growth and product synthesis. Suitable pH value can increase the reaction activity, and accelerate the utilization of glycerol and increased cell growth and product synthetic rate. In order the maximum production rate of 1,3-PD and the highest bacterial biomass, the pH gradient we selected to study the fermentation optimum pH was 5 to 10. The initial glycerol concentration was 50 g $\cdot$ L $^{-1}$  and fermentation 36 h, the 1,3-PD production and the bacterial biomass were detected. It was indicated in Fig. 2 that the maximum concentration of 1,3-PD was obtained in pH 8.0 media, and the productivity reached 18.53 g $\cdot$ L $^{-1}$ . At the same time, the molar conversion rate reached 0.497. But when pH was 9.0, the bacterial biomass reached the maximum, We can conclude that it is an alkali-resistant strain. The tolerance to higher pH could probably be related to the genetic characteristics because ZH-1 was isolated from micro-alkaline soil.

### 3.3 Optimum of Inoculum Concentration for *K. pneumoniae* ZH-1

In addition, the inoculum concentration also has a great impact on 1,3-PD production. The smaller inoculation concentration has lower utilization of glycerol and the 1,3-propanediol production. When the inoculum concentration was too high, most of the glycerol will be used for cell growth, the amount of glycerol for disproportionation was reduced, which led to a decline in the yield of 1,3-PD. Fig. 3 showed that the cell growth and the formation of 1,3-PD both increased but residual glycerol decreased gradually when the inoculum concentration was increased from 2 to 10%. But start from 12%, there was a steep decrease in the 1,3-PD formation activity and an increase in the residual glycerol. So, we determined that the optimal inoculum concentration was 10%.

### 3.4 RSM Fermentation Conditions Optimization of *K. pneumoniae* ZH-1

Appropriate fermentation conditions for commercial production of 1,3-PD has an important significance. Optimal temperature, inoculum concentration and pH should have a high light conversion efficiency to optimize the

productivity and make *K. pneumoniae* ZH-1 cultivation economically sustainable. RSM generates mathematical models that precisely represent the overall process of 1,3-PD fermentation from strain ZH-1. For cost-saving purpose, the final condition would be considered optimum if the operation parameters were as low as possible.

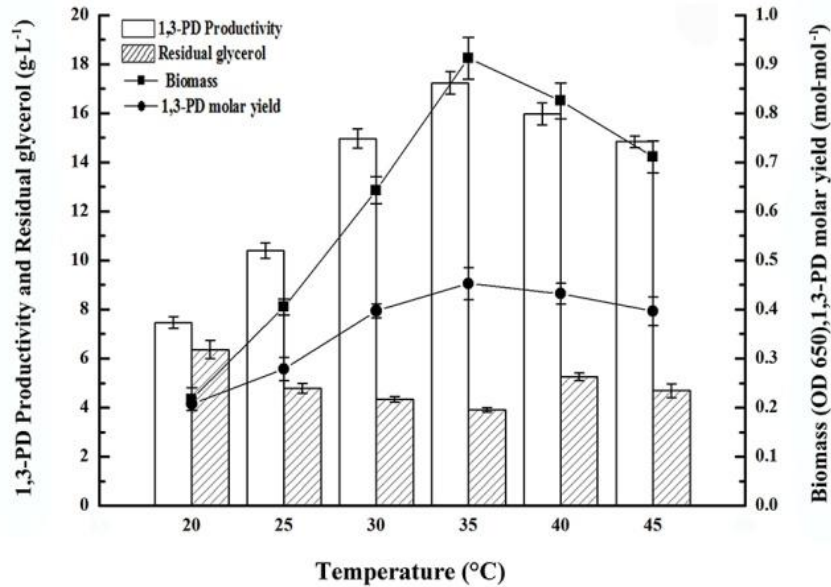


Fig. 1. Effect of temperature on biomass and product formation

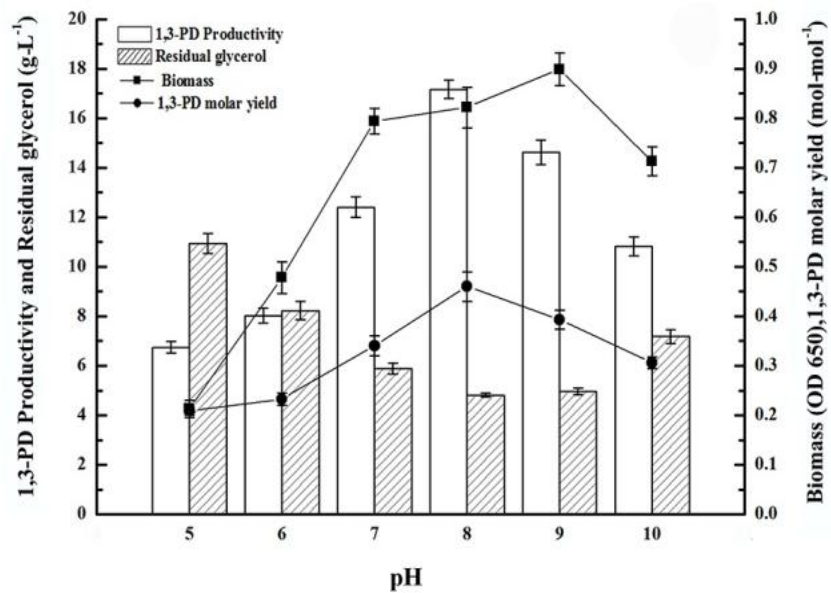
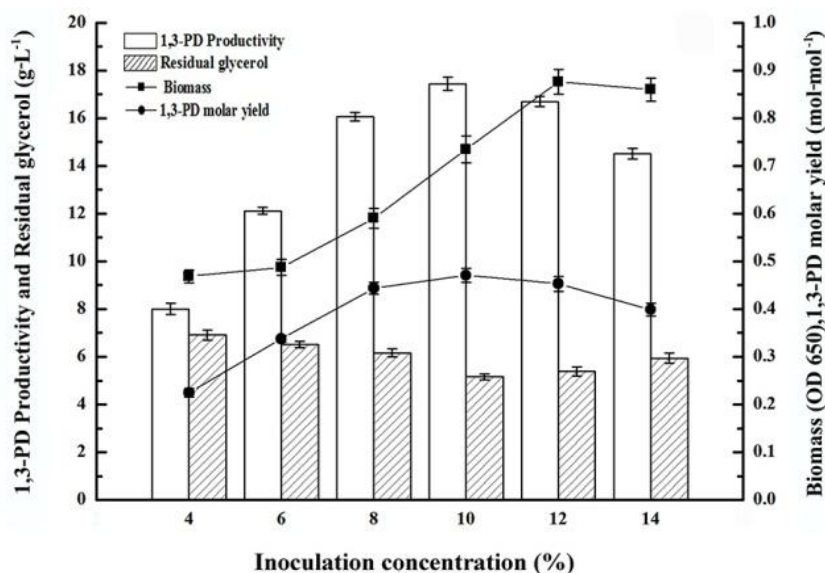


Fig. 2. Effect of pH on biomass and product formation under the optimum temperature



**Fig. 3. Effect of inoculation concentration on on biomass and product formation under the optimum temperature and pH**

According to the Box-Behnken central combination experiment principle of design, the selection the temperature, inoculum concentration, the pH value carries on three factor three levels the response surface analysis experiments (Table 1). Table 1 presents the design matrix for the experiment and the regression model proposed for response was given below:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i,j=1}^3 \beta_{ij} X_i X_j \quad (1)$$

Y is the predicted response, and  $\beta_0$  is the value of the fixed response at the central point of the experiment which is the point (0,0,0);  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are the linear, quadratic and cross-products coefficients, respectively. While demonstrating the significant effects 3-dimensional fitted surfaces were drawn. A total of 17 experiments were carried out using the RSM method. The design expert software was performed for regression and graphical analysis of data obtained. The optimum levels of temperature, inoculum concentration and pH were obtained by solving the regression equation and also analysis the response surface contour plots.

The graphical representations of the regression Eq. (2), called the response surfaces (3-D) presented in Fig. 4 was obtained using Design-Expert 8.0.6. Seventeen experimental points run randomly according to the experiment planning

(Table 1). The yield of 1,3-PD ranged from 12.54 g·L<sup>-1</sup> to 20.12 g·L<sup>-1</sup>.

The predicted model can be described by

$$Y = -403.42425 + 12.53365 \cdot X_1 + 13.55125 \cdot X_2 + 33.38675 \cdot X_3 - 0.024000 \cdot X_1 \cdot X_2 - 0.021500 \cdot X_1 \cdot X_3 - 0.097500 \cdot X_2 \cdot X_3 - 0.17087 \cdot X_1^2 - 0.57856 \cdot X_2^2 - 2.00675 \cdot X_3^2 \quad (2)$$

$R^2 = 97.71\%$  implied that the sample variation of more than 97.71% was attributed to the variables and only 2.29% of the total variance could not be explained by the model. The adjusted determination coefficient (Adj  $R^2 = 0.94770$ ) was also satisfactory to confirm the significance of the model. A lower value of coefficient of variation (CV = 3.45%) showed the experiments conducted were precise and reliable "Adeq Precision" measures the signal to noise ratio, the ratio greater than 4 is desirable. In this model the ratio of 14.878 indicates an adequate signal [18].

By applying ANOVA for the mode (Eq. (2)), the established model was found to be significant ( $P < 0.001$ ) and the lack of fit was not significant ( $P = 0.1715 > 0.05$ ), and it could be used to predict the 1, 3-PD yield. ANOVA also demonstrated that the model adequately represents the real relationship between the parameters, giving a high coefficient of determination.

The significance of each coefficient was determined by Student's t-test and *P*-value which is listed in Table 2. The larger the magnitude of t-test and smaller the *P*-value, the more significant is the corresponding coefficient [19]. The lack of fit is an indication of the failure for a model representing the experimental data at which points are not included in the regression or variations in the models cannot be accounted for random error [20]. The regression analysis of the optimization study indicated that the model terms,  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_1 \cdot X_3$ ,  $X_2 \cdot X_3$ ,  $X_1^2$ ,  $X_2^2$  and  $X_3^2$  were significant ( $P < 0.05$ ). The variable  $X_1 \cdot X_2$  was not significant ( $P > 0.05$ ). However, the interactions between the variables  $X_1 \cdot X_3$  and  $X_2 \cdot X_3$  were significant, as was shown by the low *P*-value represented in Table 2. These results indicate that the temperature and pH, inoculum concentration and pH bear some direct relationship to 1, 3-PD yield [21].

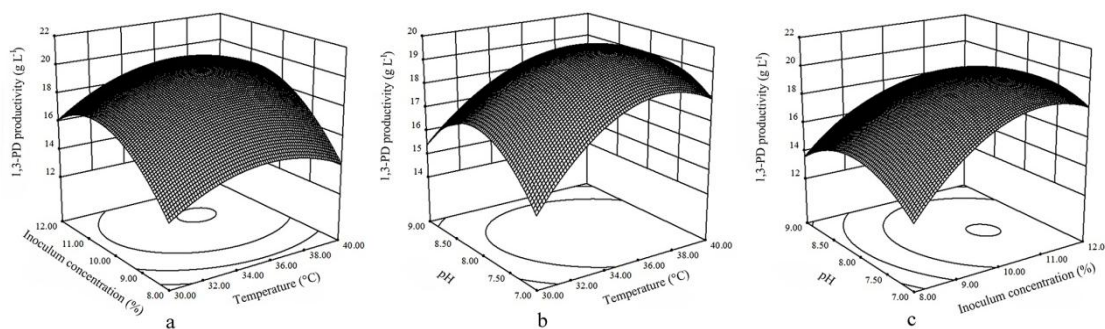
The 3D response surfaces are generally the graphical representation of the regression

equation. Fig. 4 represent the 3D plots for the optimization of culture conditions of 1,3-PD productivity. Each figure presented the effect of two variables on the production of 1,3-PD, while other variable was held at zero level. The design expert presented the optimal conditions as following: temperature 36.33°C, inoculum concentration 10.53%, pH 7.73. Under these conditions, The ZH-1 optimal fermentation conditions of correction for temperature 36°C, inoculation concentration 11% and pH 7.8. 1,3-PD production actually measured is 19.93 g-L<sup>-1</sup>, the regression model to predict the theoretical value of up to 20.1658 g-L<sup>-1</sup>, the actual measured value than the theoretical value lower 1.12%. This result demonstrate the mathematical model can well predict the relationship between the factors and yield between 1,3-PD.

After RSM optimization, the glycerol molar conversion rate reached 0.52 mol-mol<sup>-1</sup> from the original 0.34 mol-mol<sup>-1</sup>, the molar yield of 1,3-PD to glycerol was consistent with those reported

**Table 1. Experimental design with real value a of 1, 3-PD productivity**

Run	X <sub>1</sub> (°C) temperature	X <sub>2</sub> (%) inoculum concentration	X <sub>3</sub> pH	1,3-PD (g-L <sup>-1</sup> )
1	1 (40)	0 (10)	-1 (7)	17.69
2	0 (35)	1 (12)	1 (9)	15.14
3	-1 (30)	-1 (8)	0 (8)	12.54
4	1	1	0	18.69
5	-1	0	1	15.67
6	-1	1	0	16.39
7	0	1	-1	17.84
8	1	-1	0	13.89
9	0	-1	-1	13.67
10	0	-1	1	13.89
11	1	0	1	15.39
12	-1	0	-1	14.97
13	0	0	0	20.12
14	0	0	0	19.99
15	0	0	0	19.72
16	0	0	0	19.01
17	0	0	0	19.58



**Fig. 4. The 3D plots showing the effects of variables on 1,3-PD productivity. (a) The interaction of temperature and inoculum concentration. (b) The interaction of temperature and pH. (c) The interaction of inoculum concentration and pH**

**Table 2. Model coefficient estimated by multiplies linear regression**

Source	Sum of squares	df	Mean square	F value	p-value Prob > F
Model	99.75	9	11.08	33.24	<0.0001
X <sub>1</sub>	4.64	1	4.64	13.90	0.0074
X <sub>2</sub>	24.75	1	24.75	74.21	<0.0001
X <sub>3</sub>	2.08	1	2.08	6.24	0.0411
X <sub>1</sub> *X <sub>2</sub>	0.23	1	0.23	0.68	0.4379
X <sub>1</sub> *X <sub>3</sub>	2.25	1	2.25	6.75	0.0355
X <sub>2</sub> *X <sub>3</sub>	2.13	1	2.13	6.39	0.0393
X <sub>1</sub> <sup>2</sup>	12.98	1	12.98	38.93	0.0004
X <sub>2</sub> <sup>2</sup>	27.40	1	27.40	82.16	<0.0001
X <sub>3</sub> <sup>2</sup>	16.81	1	16.81	50.42	0.0002
Residual	2.33	7	0.33		
Lack of Fit	1.58	3	0.53	2.82	0.1715
Pure Error	0.75	4	0.19		
Cor Total	144.56	16			

in literatures and those previously reported [10,22-23]. YM et al. [10] studied fed-batch of a *K. pneumoniae* strain on combining biodiesel production by lipase with microbial production of 1,3-PD using a hollow fiber membrane. The molar yield of 1,3-PD to glycerol of 0.47 mol-mol<sup>-1</sup> was obtained. Another study on bioconversion of raw glycerol into 1,3-PD by *K. pneumoniae* showed that the molar yield of 1,3-PD to glycerol of 0.41 mol-mol<sup>-1</sup> [20]. In this study, the strain ZH-1 had a higher molar conversion rate than some strains of the previous study, whereas compared with the study of Yang et al. [24] (0.62 mol-mol<sup>-1</sup>). The 1,3-PD conversion rate of strain ZH-1 should be further improved, and we will have further research on the conversion of ZH-1 by adding the metabolites and some other methods.

#### 4. CONCLUSION

This work showed that the alkali-resistant *K. pneumoniae* ZH-1 has the potential for 1,3-PD production at high efficiency under anaerobic conditions. The optimal conditions of 1,3-PD production as follows: temperature 36°C, inoculum concentration 11%, pH 7.8. Under these conditions, the practical yield of 1,3-PD was 19.93 g-L<sup>-1</sup> and the molar yield was 0.52 mol-mol<sup>-1</sup>. Compared with 1,3-PD yield from other strains, the strain *K. pneumoniae* ZH-1 has a higher pH tolerance and a higher molar conversion rate. To provide more data necessary to establish technically and economically feasible process, further investigation is needed.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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