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Response Surface Modeling and Optimization of Culture Media in Fermentative Production of 2,3-butanediol

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ABSTRACT

2,3-Butanediol is a promising platform chemical with extensive industrial applications.The present study aims to develop a microbial fermentation approach for the production of 2,3-Butanediol. A wild strain of bacteria,*Enterobacter cloacae* SG1,was studied its capacity to ferment glucose to 2,3- butanediol. Batch fermentation parameters were optimized and media engineering was performed using statistical approach by response surface methodology for increasing the yield of 2,3-Butanediol. At the optimized condition, 25.86 g/L 2,3-butanediol could be obtained from Box-Behnken design with 0.36g BDO/g of glucose. The optimization resulted in almost two times increase in the production of 2,3-Butanediol.

1. Introduction

2,3 Butanediol (2,3-BDO) is a platform chemical, extensively used in food,cosmetics,paints,solvents,coating, pharmaceutical and adhesive industries (Ji et al., 2011). Apart from these applications the dehydration product, methyl ethyl ketone (MEK) is a valuable fuel additive in aviation fuels (Cui et al., 2018). Another dehydration product 1,3-butadiene is a precursor for synthetic rubber (Duan et al., 2015). Derivatives like acetoin and diacetyl are widely used as a flavoring agent used in dairy products and fermented foods (Xiu and Zeng, 2008). Currently, commercial synthesis of 2,3-BDO is through petrochemical route in which hydrolysis of 2,3-butene oxide is carrying out at high temperature and pressure via a number of catalytic reactions(Song et al., 2018). The biological production of this chemical offers environmental advantageous and it also makes a sustainable way of production.

Various microbial strains such as *Bacillus subtilis* (Deshmukh et al., 2015), *Bacillus licheniformis* (Song et al., 2018),*Paenibacillus polymyxa* (Yu et al., 2011), *Serratia marcescens* (Shi et al., 2014), *Klebsiella oxytoca*(Ji et al., 2010), *Klebsiella pneumonia* (Petrov and Petrova, 2009)and different *Enterobacter*(Jung et al., 2012) species are reported to produce BDO from different carbon sources. 2,3-BDO is produced in bacteria by a mixed acid pathway and the known substrates are different monosaccharides (hexoses and pentoses) (Biakowska, 2016), disaccharides (sucrose, lactose), glycerol and biomass-derived sugars. The 2,3-BDO fermentation can be performed either anaerobically or micro-aerobically (Byun et al., 1994). Different fermentation strategies like batch and continuous modes have been reported with improved 2,3-BDO production.

Increased production cost is one of the major hindrances in any industrial fermentation. Media cost and substrate cost accounts for a significant percentage of production cost (Koutinas et al., 2016). One way to address this issue is to improve the product yield. The improvement of fermentation efficiency with maximum yield could be achieved by media engineering and through process parameters optimizations. Statistical tools have a definite role in industrial bioprocess by achieving higher titers of targeted product with minimal cost. Response surface methodology (RSM) is successfully used in media optimization for improved production of platform chemicals.

RSM is a statistical tool employed to maximize the production of a special substance by optimization of operational factors. Box Behnken (BB) design is one of the RSM approaches which is used for the development of optimum processes with precise conditions and for efficient screening of process parameters(Behnken, 1960).

Enterobacter cloacae SG1 is a gram-negative rod-shaped bacteria isolated from rice cultivating fields of southern Kerala, India. It is reported to produce 2,3-BDO from glucose and acid pretreated liquor of oil palm biomass (Hazeena et al., 2016). This study aims to focus on the substrate and product derived inhibition on the growth of *E.cloacae* SG1 and media optimization for improved production of 2,3-BDO via response surface

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methodology.

2. Materials and methods

2.1. Media and chemicals

(2S, 3S) - (+), (2R, 3R) - (“)-and meso-2,3BDO and acetoin (>98%) were purchased from Merck (Germany). All other chemicals were of analytical grade available commercially.

2.2. Bacterial strain maintenance

The *Enterobacter cloacae* SG1 was maintained as 20% glycerol stocks and regularly sub-cultured in nutrient agar plates. The bacterial strain was cultured in seed medium consist of peptone 10 (g/L), beef extract 10 (g/L) and sodium chloride 5 (g/L). The 24-hour old seed inoculum was transferred to fermentation media.

2.3. Substrate and product derived inhibition on growth of E. cloacae *SG1*

Fermentation media contains yeast extracts 5.0 (g/L), K_2HPO_4 14 (g/L), KH_2PO_4 6.0 (g/L), $(NH_4)_2SO_4$ 2.0 (g/L), sodium citrate dehydrate 1.0(g/L) and $MgSO_4.7H_2O$ 0.2 (g/L). Different initial glucose concentrations such as 60, 80,100 and 120 g/L were selected for substrate inhibition studies. For product inhibition experiments 10, 20, 30 and 40g/L of 2,3-BDO were added to the media after 24 hours of incubation time. The initial pH of the media was set as 6.5. Fermentation was carried out at 37^0C, 200rpm for 96 hours with 2% (v /v) inoculum where each 1ml inoculum contained 7×10^9CFU (Colony Forming Units) and the total reaction volume was 100mL in 250mL conical flasks.

2.4. Optimization of organic nitrogen source for the production of BDO

For the optimization of organic nitrogen source,different commercial as well as cheaply available substrates, known for nitrogen content,were tested. Meat extracts (ME), beef extracts (BE), yeast extracts (YE), sesame oil cake (SOC), coconut oil cake (COC), groundnut oil cake (GOC), peptone(PEP) and corn steep liquor (CSL) were added to fermentation media at a concentration of 5g/L.

2.5. Response surface methodology- Box Behnken design

Statistical software package Minitab 17.1.0 (Minitab Inc., USA) was used to create suitable experimental designs and for the analysis of the results. Box-Behnken (BB) design was used in this study to optimize the concentration of significant parameters for improving 2,3-BDO production. These parameters include glucose,CSL, and sodium citrate. Each factor in the design have a 3 level values and the total number of experimental runs were 15. All experiments were performed in duplicates, and the mean value of BDO production was taken as the response. The surface and contour plots were analyzed according to the response and validation of the model was performed under the conditions predicted by the model in triplicates.

2.6. Analytical methods

Bacterial growth was determined by taking the optical density (OD) values at 620nm using UV-visible spectrophotometer (UV-1601, Shimadzu, Japan). The concentration of 2,3-BDO and acetoin was determined by HPLC (Shimadzu, Japan) equipped with RI detector and Rezex-ROA organic acid (300x7.8mm Phenomenex) column at 65^0C using 0.01 N H_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min.

3. Results and discussion

Microbial 2,3-BDO production has a history of more than 100 years. It was first investigated in 1906 by Harden and Walpole and in 1912 by Harden and Norris (Magee and Kosaric, 1987) using *Klebsiella pneumoniae.* After this several microbe were reported to produce 2,3-BDO. In the present study, *Enterobacter cloacae* SG1, a gram-negative rod-shaped bacteria, was used for the production of 2,3-BDO. This bacteria was isolated from rice cultivating fields of southern Kerala, India, and it produces 2,3-BDO from glucose and acid pretreated liquor of oil palm biomass (Hazeena et al., 2016).

3.1. Substrate inhibition on growth of E. cloacae SG1

In order to find the effect of substrate inhibition and also to understand the effect of concentration of glucose on growth and 2,3-BDO production by this bacterial strain, various glucose concentrations were selected in the cultivation media. Figure 1 depicts the effect of glucose concentration on the growth of *E.cloacae* SG1 in terms of optical density at 620nm. When the initial glucose concentration increased from 60 g/L to 80 g/L, after 24 hours of incubation, there was a clear decline in the growth (OD inhibition on growth and 2,3-BDO production by *E.cloacae* SG1

3.2. Product inhibition on growth of E.cloacae *SG1*

End product inhibition is also an issue to address in microbial fermentation processes. In order to study the effect of concentration of 2,3-BDO on the metabolic state of *E. cloacae* SG1, the organism was cultivated in the presence of externally added 2-3-BDO at concentrations 10-40 g/L. As shown in Figure 2, the presence of higher amount of 2,3-BDO in the fermentation broth has a negative impact on the growth of microorganism which in turn affects the growth of organism as visible in the optical density values. The control experiment where there is no 2,3-BDO was added externally, showed higher growth than other test samples which were supplemented with an extra amount of BDO. As the concentration of the BDO increases, there showed a retarded growth within 24 hours of the incubation period. The possible explanation for 2,3-BDO inhibition to bacterial growth was the decrease in water activity(Fond.etal, 1985). Compared to other acids of mixed acid pathway 2,3-BDO is a neutral product and not a strong inhibitor.

3.3. Optimization of organic nitrogen source for the production of BDO

Among the eight different organic nitrogen supplemented to the fermentation media, YE,GOC, and CSL showed higher BDO production (Figure 3) and the 2,3-BDO concentrations were 12.62, 13.87 and 14.03 g/L respectively. Since CSL gave the highest 2,3-BDO production,it was selected as the nitrogen source for further studies. Replacing the costly nutrients like yeast extracts and meat extract with cheaper nutrient

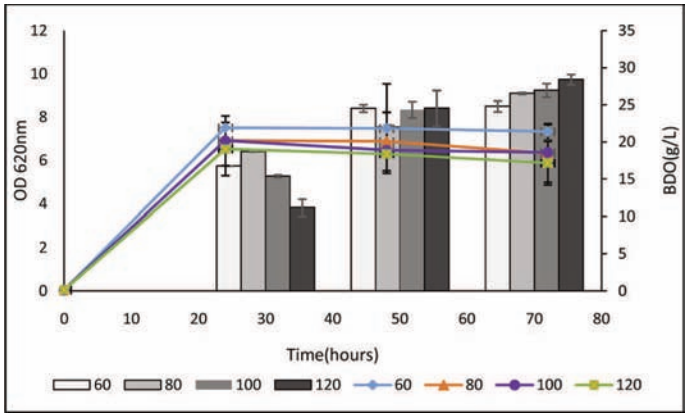

Figure 1: Effect of glucose concentration on growth and production of 2,3-BDO by *E. cloacae* SG1

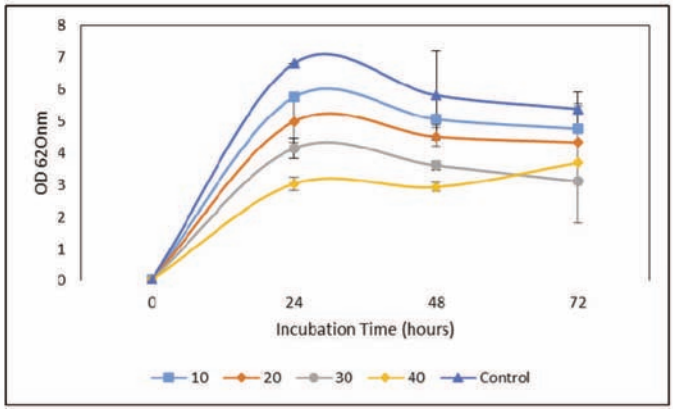

Figure 2: Product inhibition analysis of 2,3-BDO concentration on the growth of *E. cloacae* SG1

dropped from 7.51 to 6.91 units). The consecutive increase of initial glucose concentration from 80 to 100 and 120g/L clearly lowered the growth in terms of optical density as well as visible turbidity within 24hours of incubation time. After 72 hours of incubation 2,3-BDO concentrations found for different initial sugar concentrations 60,80,100 and 120 (g/L) were 24.80,26.56,26.97 and 28.41 respectively. The respective yields were 0.41,0.33, 0.26 and 0.23. A reduction in the yield was noted above 60g/L which clearly indicated the substrate derived

Table 1: Box-Behnken experimental design matrix with BDO yield

Run order	Glucose (g/L)	CSL (g/L)	Sodium citrate(g/L)	Experimental BDO yield (g/L)	Predicted BDO yield (g/L)
1	70	6	3	9.73	11.70
2	60	8	1	25.03	24.06
3	70	4	2	6.60	3.72
4	50	4	2	2.49	3.20
5	60	4	3	3.00	4.08
6	50	8	2	14.76	17.58
7	50	6	1	11.04	9.60
8	60	6	2	11.98	13.15
9	50	6	3	11.14	9.54
10	70	8	2	25.86	25.10
11	60	6	2	12.98	12.81
12	70	6	1	13.63	15.51
13	60	4	1	3.82	4.89
14	60	8	3	21.83	20.87
15	60	6	2	13.31	12.87

supplement can reduce the process cost to a significant extent (Koutinas et al., 2016). CSL cost is 0.5-0.7 US $/Kg while YE cost is almost ten times higher than that of CSL (7.8-8 US$/Kg).CSL is a low-cost nitrogen source suitable with industrial fermentation with a good amount of water-soluble vitamins, amino acids and minerals(Yang et al., 2013). Previous reports show that yeast extract,urea, and ammonium salts improve 2,3-BDO yield because the synthesis of protein is a constant function of cellular metabolism. Since nitrogen is a major component of protein, nitrogen must be supplied in large quantities. Yeast extract is a frequently used nitrogen source for high 2,3-BD production (Laube et al., 1984a, Laube et al., 1984b). However, the high cost of yeast extract prohibits its utilization in large quantities for commercial processes (Xiao-Jun et al 2011).Hence in the present study, replacing costly yeast extract with cheaper CSL could make the process more economical.

3.4. Response Surface Methodology (RSM)

Multiple regression analysis was used to analyze the data and thus a polynomial equation was derived from regression analysis as follows.

$$Y= -44.11 + 1.30\ X_1 - 2.73\ X_2 + 7.58\ X_3 - 0.01\ X_1^2 + 0.21\ X_2^2 - 0.19\ X_3^2 + 0.09\ X_1X_2 - 0.10\ X_1X_3 - 0.30\ X_2X_3$$

Where X_1, X_2 and X_3 are glucose, CSL and sodium citrate concentrations respectively and Y is the 2,3-BDO yield.

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results are shown in Table 2. The model F value of 11.34 implies the model is significant and also shows that there is a 0.8% chance that the model F value could occur due to noise. The R^2 value (multiple correlation coefficient) closers to 1 denote a better correlation between the observed and predicted values. In this case, the value of R^2 (0.953) indicates a good correlation between the experimental and predicted values. The P values denote the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. The P values suggest that linear interactions of factors are significant.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface curves are represented in Figures 4 a,b & c. Figure 4a represents the interaction between glucose and CSL. It is evident from the figure that maximum 2,3-BDO production was at glucose concentration above 60 g/L and CSL concentration of 8 g/L. As the CSL concentration is decreasing, there is a decrease in 2,3-BDO production. The graph clearly shows the interaction effect of both these media components on 2,3-BDO production. Figure 4b shows the interaction between glucose and sodium citrate. Maximum 2,3-BDO production was observed at a glucose concentration of 70 g/L. Sodium citrate was found to have a negative effect on 2,3-BDO production. As the concentration of 2,3-BDO increases at 70g/L glucose, there is a decrease in BDO production. Figure 4c represents the surface and contour plot showing the effect of the interaction of CSL and sodium citrate. CSL concentration of 8g/l was found to be best at this condition with 1 g/L sodium citrate. The low level of sodium citrate concentration during fermentation was observed in this case also.

An optimization plot of 2,3-BDO production is shown in figure 5. The plot predicts the maximum 2,3-BDO can be achieved at the conditions selected for the present experimental conditions. Based on the analysis,

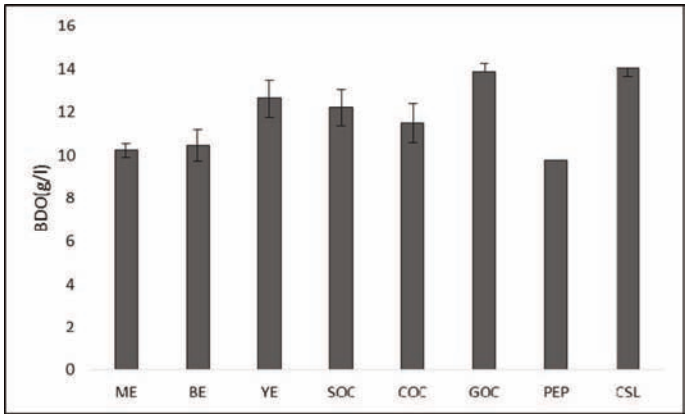

Figure 3: Effect of different organic nitrogen source on 2,3-BDO production

Table2: ANOVA of Box Behnken design

Sl No.	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1	Regression	9	707.624	707.624	78.625	11.34	0.008
2	Linear	3	681.435	681.435	227.145	32.77	0.001
3	Square	3	8.562	8.562	2.854	0.41	0.752
4	Interaction	3	17.627	17.627	5.876	0.85	0.524
5	Residual error	5	34.658	34.658	6.932		
6	Lack-of-Fit	3	33.707	33.707	11.236	23.04	0.041
7	Pure error	2	0.951	0.951	0.475		
8	Total	14	742.282				

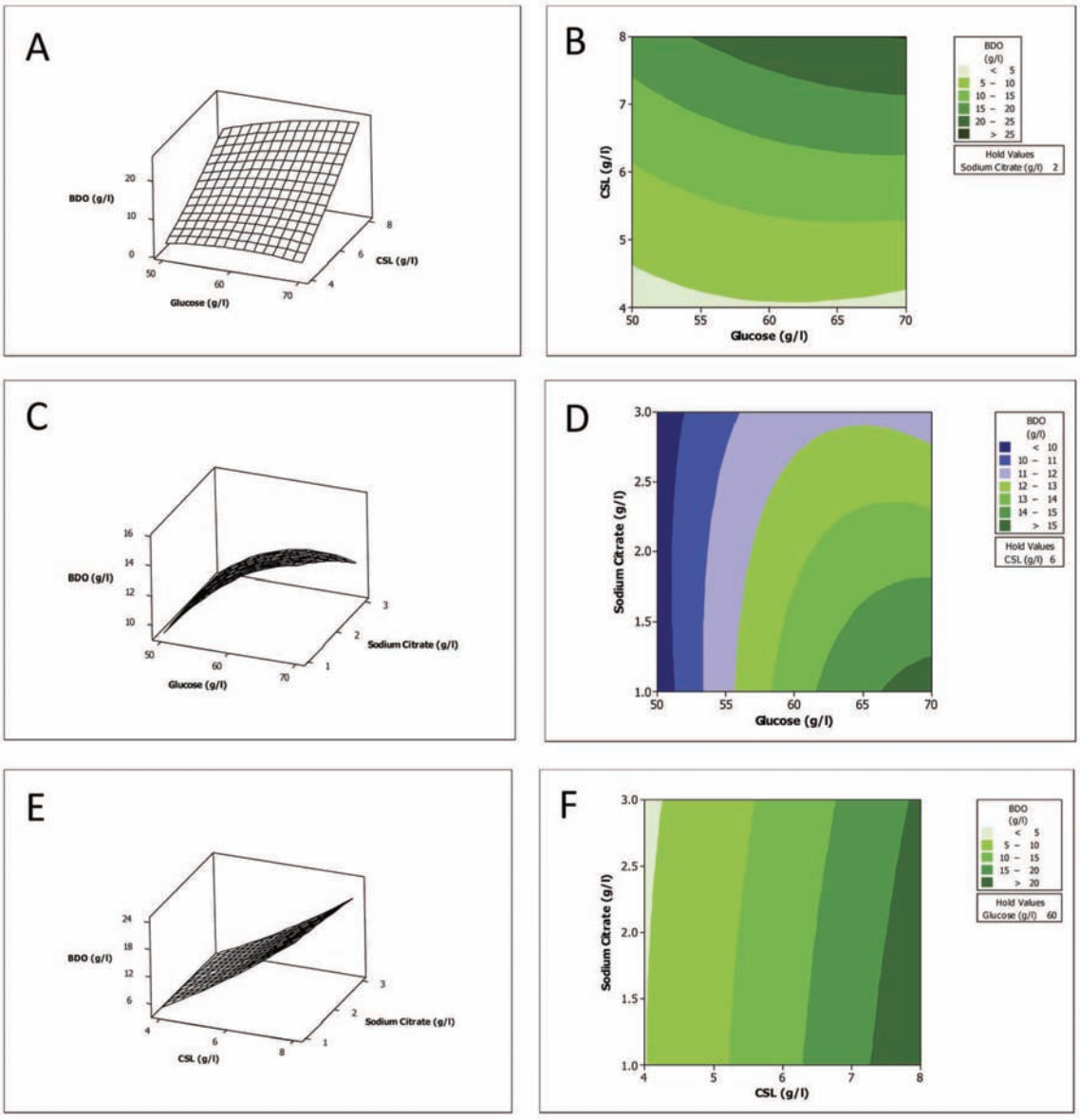

Figure 4: Response Surface Methodology optimization plots for 2,3-BDO production (A) surface plot showing the effect of CSL and glucose; (B) contour plot showing the effect of CSL and glucose; (C) surface plot showing the effect of sodium citrate and glucose; (D) contour plot showing the effect of sodium citrate and glucose; (E) surface plot of showing the effect of sodium citrate and CSL; (F) contour plot showing the effect of sodium citrate and CSL

the software predicts the maximum 2,3-BDO production as 27.5g/L at a glucose concentration of 70 g/L, CSL of 8 g/L and sodium citrate 1 g/ Lwith a biomass yield of 49.3 mg/L of dry cell weight. (Data not shown).In order to confirm the above details for the maximum production of 2, 3-BDO, the validation experiment was carried out. The correlation coefficient obtained for this analysis was 0.906.

Among bacteria, *Klebsiella, Enterobacter, Bacillus*, and *Serratia*, are considered of industrial importance in the production of 2,3-BDO (Maddox, 1996). *Klebsiella* sp. has been demonstrated to be potentially applicable in the industrial production of 2,3-BDO. However, these microbes are an opportunistic pathogen and hence it limits its industrial application. In light of this, *Enterobacter* sp. is a promising candidate for industrial use. Optimization of the culture medium is a very important aspect in any microbial bioprocess development. Several strategies have been widely used to enhance 2,3-BDO production, such as optimizing medium component, optimizing fermentation operating conditions and establishing mathematical models, etc. (Celinska and Grajek 2009) and these resulted improvement in the 2,3-BDO yield (Yang et al. 2012). The present study resulted in two times increase in the 2,3-BDO production using Box Behnken optimization by *Enterobacter cloacae* SG1 under batch fermentation condition.

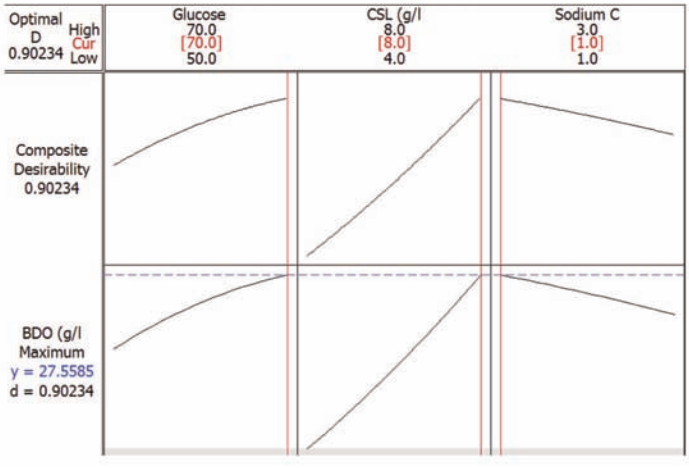

Figure 5: Optimization plot of 2,3-BDO production

4. Conclusion

Substrate and product derived inhibition on growth and 2,3-BDO production by *Enterobacter cloacae* SG1 was investigated. Various organic nitrogen sources were screened for 2,3-butanediol production and corn steep liquor was found to be effective. From response surface methodology analysis the concentrations of glucose, corn steep liquor, and sodium citrate were optimized as 70g/L, 8g/L, 1g/L respectively for 2,3-butanediol production using *Enterobacter cloacae* SG1. The experimental model was validated and found to be fitting.The optimization resulted in two times increase in the production of 2,3-BDO.

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