PRODUCTION OF 2,3-BUTANEDIOL BY *KLEBSIELLA PNEUMONIAE* FROM ENZYMATIC HYDROLYZATE OF SUGARCANE BAGASSE

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Fermentation conditions for 2,3-butanediol (2,3-BD) production by *Klebsiella pneumoniae* CGMCC1.9131 were optimized statistically in shake flasks. Four significant factors including the initial concentrations of yeast extract, glucose, K_2HPO_4, and $(NH_4)_2SO_4$ were optimized by Response Surface Methodology (RSM). To further improve the yield of 2,3-BD, EDTA Na_2 was added to the medium. After optimization, the yield of 2,3-BD was 0.44 g/g glucose and the final concentration was 26.20 g/L when initial glucose concentration was 60 g/L. The enzymatic hydrolyzate of pretreated sugarcane bagasse by alkali-peracetic acid (PAA) and dilute acid were further used as feedstock to produce 2,3-BD under the optimized conditions, and the yields of 2,3-BD were 0.36 and 0.43 g/g consumed sugars, respectively. The experimental results indicated that the enzymatic hydrolyzate could be well converted to 2,3-BD

Keywords: 2,3-butanediol; Klebsiella pneumoniae; Hydrolyzate; Sugarcane bagasse

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INTRODUCTION

Most organic chemicals available in bulk quantities are currently produced from petrochemical feedstocks derived from crude oil or natural gas. However, due to the shortage of fossil oil supplies and rising petroleum prices, alternative ways should be developed. Bio-refinery systems that integrate biomass conversion processes and equipment to produce fuels, power, and chemicals from renewable resources, underlying the biotechnology, have become an emergent area of intellectual endeavor and industrial practice with great promise and potential to reduce greenhouse gases emission (Kamm and Kamm 2004; Lee *et al.* 1999; Ragauskas *et al.* 2006). Many chemicals, which could only be produced by chemical processes in the past, now have the potential to be generated biologically from renewable resources (Danner and Braun 1999; Hatti-Kaul *et al.* 2007; Ragauskas *et al.* 2006). Microbial production of 2, 3-butanediol (2,3-BD) is one of the examples. 2,3-Butanediol is a bulk chemical (<1 $/kg) (Celinska and Grajek 2009) that exhibits a wide range of potential utilizations in chemicals, medicines, printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, as well as cosmetics (Jordan *et al.* 2001; Romano *et al.* 2003; Soltys *et al.* 2001; Xiu and Zeng 2008).

Microorganism species including those belonging to the genera *Klebsiella*, *Enterobacter*, *Bacillus*, *Paenibacillus*, *Serratia*, and *Aeromonas* can produce 2,3-BD from different feedstocks (Celinska and Grajek 2009; Garg and Jain 1995; Magee and Kosaric 1987; Syu 2001). Different microorganisms produce different stereoisomers of 2,3-BD (three stereoisomers exist: dextro-[L-(+)-] , levo-[D-(−)-], and meso forms), and a mixture of two stereoisomers is generally formed (Celinska and Grajek 2009; Ji *et al.* 2011). Recently, there has been increased interest in the production of 2,3-BD by microbial fermentation (Celinska and Grajek 2009; Wu *et al.* 2008; Ji *et al.* 2009a,b). Most studies on the production have focused on the use of pure substrates, such as glucose as the most common feedstock (Syu 2001; Qin *et al.* 2006). However, the major cost in most biomass conversion processes appears to be the substrate cost (Voloch *et al.* 1985), and the price of the final product is mostly affected by the raw material cost (Willke and Vorlop 2007). So production of 2,3-BD from inexpensive lignocellulose hydrolyzate might be beneficial to reduce the production cost. One of the most important and available lignocellulosic biomass in tropical countries is sugarcane bagasse, the fibrous residue obtained after extracting the juice from sugarcane in the sugar production process. It has been estimated that about 5.4×10^8 dry tons of sugarcane are processed annually throughout the world (Cardona *et al.* 2010).

In our previous work, *Klebsiella pneumoniae* CGMCC 1.9131, which can produce 1,3-propanediol (1,3-PD) with high yield when glycerol is used as a carbon source, has been isolated (Hao *et al.* 2008). This strain has been successfully used for 1,3-PD production in industrial scale by China Hunan Rivers Bioengineering Co., Ltd.. Moreover, it also has a strong ability to produce 2,3-BD when sugars are used as a carbon source. However, the optimum fermentation for 2,3-BD production by this strain has not been available. Therefore, in the present study, we employed the response surface methodology (RSM) to optimize the medium compositions for 2,3-BD production. To further improve the production of 2, 3-butanediol, EDTA Na_2 was added to the medium, and the concentration was optimized. Then the enzymatic hydrolyzate of pretreated sugarcane bagasse was used as feedstock for 2,3-BD production under the optimized conditions.

EXPERIMENTAL

Microorganism

Klebsiella pneumoniae CGMCC 1.9131 was isolated from soil in our lab, according to Hao *et al.* (2008). The strain is now stored in China General Microbiological Culture Collection Center (CGMCC, Beijing, China). It has been proved that this strain is more capable than other strains for 2,3-BD production according to our previous work (Song *et al.* 2011).

Growth Medium and Culture Conditions

K. pneumoniae CGMCC 1.9131 was maintained on agar slants containing the following medium: glucose 15 g/l, peptone 10 g/l, KCl 5 g/l, yeast extract 5 g/l, and agar

at pH 7.0. The slants were incubated at 37°C for 12 h and then stored at 4°C (Ma *et al.* 2009).

The inocula were prepared by inoculating a full loop of cells from freshly prepared slants into 100 mL of the following medium: glucose 20 g/L, $(NH_4)_2HPO_4$ 5 g/L, KCl 1 g/L, $MgSO_4$ 0.3 g/L, pH 7.0. The cultivation was conducted in 250-mL shake flasks for 12 h with agitation (160 rpm, reciprocal shaker) at 37°C. 1.5% (v/v) of inocula was added aseptically to the 250 mL flask with 100 mL fermentation medium containing: yeast extract 6 g/L, glucose 60 g/L, $(NH_4)_2SO_4$ 7 g/L, K_2HPO_4 5 g/L, KCl 0.4 g/L, $MgSO_4$ 0.1 g/L, $FeSO_4 \cdot 7H_2O$ 0.02 g/L, $MnSO_4 \cdot 7H_2O$ 0.01 g/L, and $ZnSO_4$ 0.01 g/L at pH 7.0. The fermentation conditions are as follows: 37 ^oC, 150 rpm in an air-bath shaker.

Analytical Methods

Biomass concentration was measured by the optical density (OD) calibration curved determined at 600 nm, and cell dry weight was calculated according to standard curve. Glucose, 2,3-BD, lactate, succinic acid, acetic acid, and ethanol were determined with a Shimadzu 10AVP HPLC system (Shimadzu Corp., Kyoto, Japan) with an Aminex HPX-87H column ($300 \times 7.8 mm^2$) (Bio-Rad, Palo AHO, CA) and an RID-10A refractive index detector.

The mobile phase was 0.005 mol/L H_2SO_4 solution at 0.8 mL/min. The column temperature was controlled at 65 ^oC. The culture samples were prepared by centrifuge at 10,000 g for 5 min to pellet the cell, followed by filtration using nylon syringe filters (pore size 0.45 mm, TaKaRa) and dilution with deionized water before analysis (Zheng *et al.* 2008).

Statistical Experimental Design

Compared with conventional methods, Response Surface Methodology (RSM) is a time and labor saving method which also reveals the interaction between the components of a medium and seeks the optimum levels (Tang *et al.* 2004; Ghadge and Raheman 2006; Zheng *et al.* 2008). Different levels for one numeric factor are assigned corresponding to different design. RSM consists of the central composite design (CCD), the box-behnken design (BBD), the one factor design, the D-optimal design, the user-defined design, and the historical data design. In the experiment, central composite design was used to optimize the initial concentrations of yeast extract, glucose, K_2HPO_4, and $(NH_4)_2SO_4$ of the medium.

A two-step experimental design was used to optimize the medium for 2,3-BD production. CCD (Kennedy and Krouse 1999), an efficient technique for medium-component optimization, was selected to show the statistical significance of the effects of yeast extract, glucose, $(NH_4)_2SO_4$, and K_2HPO_4 on the concentration of 2,3-BD by *K. pneumoniae* CGMCC1.9131. The experiments were designed by using the Design Expert 8.0.5 Trial (State Ease Inc., Minneapolis, MN, USA). This approach makes it possible to estimate the second degree polynomial of the relationships between the factors and the dependent variable and gives information about interaction between variables (factors) in their relation to the dependent variable in CCD. The ranges of the variables were selected according to the preliminary single-factor experiments. The levels of variables are shown in Table 1.

Table 1. Levels of Variables Used in the Experimental Design

Variables	Range and levels				
	-2	-1	0	+1	+2
X_1, Yeast extract（g·l^{-1}）	2	4	6	8	10
X_2, Glucose（g·l^{-1}）	20	40	60	80	100
X_3, （$NH_4)_2SO_4$（g·l^{-1}）	3	5	7	9	11
X_4, K_2HPO_4（g·l^{-1}）	3	5	7	9	11

For the optimization of the culture conditions, a 24 factorial central composite design with eight star points and six replicates at the center points leading to 30 runs was employed. For statistical calculations, the relationships between the coded values and actual values are described by the following equation,

$$x_i = \frac{(X_i - X_0)}{\Delta X} \qquad i=1, 2,..., \ \kappa \tag{1}$$

where x_i is the dimensionless coded value of the independent variable X_i; X_i is the actual value of that independent variable; X_0 is the real value of the independent variable X_i at the center point, and ΔX is the step change. The second-order polynomial, Eq. (2), is applied to explain the role of each variable, their interactions, and statistical analysis to obtain predicted yields,

$$C_i = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i,j=1}^{4} \beta_{ij} x_i x_j \tag{2}$$

where C is the predicted response, concentration of 2,3-BD (g/l) after 48 hours' fermentation; β_0 is the offset term; β_i is the linear effect; β_{ii} is the squared effect; β_{ij} is the interaction effect, and x_i and x_j are the input variables. Each experiment was performed in triplicate.

The design expert software was used for regression and graphical analysis of data obtained. The optimum levels of yeast extract, glucose, $(NH_4)_2SO_4$, and K_2HPO_4 concentrations were obtained by solving the regression equation and also analyzing the response surface contour plots.

Preparation of Enzymatic Hydrolyzate of Sugarcane Bagasse

Raw materials

Sugarcane bagasse used in the present work contained 1.38% ash, 5.16% hot water extractives, 34.20% NaOH (1 wt% solution) extractives, 3.17% benzene-ethanol extractives, 44.98% cellulose, 76.76% holocellulose, 18.45% Klason lignin, 1.80% acid-soluble lignin, and 2.62% acetyl group, as shown in our previous work (Zhao *et al.* 2011). The cellulase used for hydrolyzing pretreated bagasse was NS 50013 (～40 FPU/mL of cellulase complex activity, 28 CBU/mL of ß-glucosidase, and ~55 U/mL xylanase activity) produced by Novozymes. The supplemental *β*-glucosidase (NS 50010) was also produced by Novozymes with the enzyme activity of 245 CBU/mL.

Pretreatment and enzymatic hydrolysis of sugarcane bagasse

Alkali-peracetic acid (PAA) pretreatment and dilute acid pretreatment were employed to increase the enzymatic digestibility of sugarcane bagasse according to our previous work (Zhao *et al.* 2011). Alkali–PAA pretreatment was carried out in a 1000 mL glass flask immerged in a water bath. Bagasse was first treated with 10% NaOH (based on raw material) at 90 ^{o}C for 1.5 h with a 3:1 liquor-to-solid ratio (L/kg). The pretreated solid was washed with water to neutrality and then filtered until liquid content of solid phase was about 75%. The solid was further treated by an oxidative delignification stage with PAA loading of 15% (based on raw material) at 75 ^{o}C for 3 h. The pulp was washed with water until neutrality and stored in valve bags at 4 ^{o}C for further analysis and enzymatic hydrolysis. It was determined that the glucan, xylan, and lignin contents of the obtained alkali–PAA pulp were 78.5%, 20.1%, and 1.04%, respectively. Sugarcane bagasse was also pretreated by dilute acid (1% sulfuric acid) at 160 ^{o}C with 10:1 (w/v) liquid-to-solid ratio for 30 min in a 5 L autoclave, and the heating-up time was 1.5 h. Mechanical stirring with 300 rpm rotation speed was employed in order to keep the mixture as homogenous as possible. The pretreated solid (DAPB) was washed with water until neutrality and stored in valve bags at 4 ^{o}C for further analysis and enzymatic hydrolysis. The main components of DAPB were determined as 56.0% glucan, 2.4% xylan, and 40.6% lignin.

The alkali-PAA pretreated bagasse was then hydrolyzed at 50 ^{o}C, pH 4.8 (0.1 M sodium acetate buffer) with cellulase and supplemental β-glucosidase of 15 FPU and 10 CBU/g solid at an initial solid (total solid, DW) consistency of 10%. The conditions for enzymatic hydrolysis of dilute acid-pretreated bagasse were the same to that of alkali-PAA pretreated bagasse, except that the initial solid consistency was 15%. In the experiments, sugar concentrations were determined by HPLC.

RESULTS AND DISCUSSION

Optimization of Fermentation Medium

The experimental design and results are shown in Table 2. By applying multiple regression analysis to the experimental data, the following second-order polynomial equation (Eq. 3) was obtained to explain 2,3-BD production. Results of the corresponding analysis of variance (ANOVA) and significance tests of regression coefficient are shown in Tables 3 and 4, respectively.

$$C = 13.75 - 1.38X_1 - 0.19X_2 - 0.36X_3 + 0.029X_4 - 0.14X_1^2 - 1.25X_2^2 - 0.64X_3^2 - 0.16X_4^2$$
$$+0.0.035X_1X_2 + 0.055X_1X_3 - 0.62X_1X_4 - 0.32X_2X_3 + 0.096X_2X_4 - 9.188E-03X_3X_4 \quad (3)$$

The Model F-value of 19.23 implies the model was significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The goodness of fit of the model was examined by determination coefficient (R^2 = 0.9472), which implied that the sample variation of more than 94% was attributed to the variables, and only 5.28% of the total variance could not be explained by the model.

Table 2. Results of the Central Composite Experiment

Runs	X_1	X_2	X_3	X_4	C ($g \cdot L^{-1}$)	Runs	X_1	X_2	X_3	X_4	C ($g \cdot L^{-1}$)
1	-1	-1	-1	-1	12.51±0.12	16	1	1	1	1	9.59±0.10
2	1	-1	-1	-1	11.48±0.08	17	-2	0	0	0	16.17±0.22
3	-1	1	-1	-1	12.66±0.11	18	2	0	0	0	9.80±0.08
4	1	1	-1	-1	11.31±0.11	19	0	-2	0	0	8.10±0.07
5	-1	-1	1	-1	12.99±0.12	20	0	2	0	0	9.01±0.10
6	1	-1	1	-1	11.92±0.10	21	0	0	-2	0	12.13±0.13
7	-1	1	1	-1	11.41±0.11	22	0	0	2	0	9.89±0.10
8	1	1	1	-1	9.58±0.11	23	0	0	0	-2	12.41±0.20
9	-1	-1	-1	1	14.02±0.21	24	0	0	0	2	13.43±0.21
10	1	-1	-1	1	9.98±0.09	25	0	0	0	0	13.63±0.18
11	-1	1	-1	1	13.8±0.12	26	0	0	0	0	14.00±0.28
12	1	1	-1	1	9.56±0.09	27	0	0	0	0	13.89±0.16
13	-1	-1	1	1	13.92±0.12	28	0	0	0	0	13.48±0.14
14	1	-1	1	1	9.54±0.09	29	0	0	0	0	13.72±0.17
15	-1	1	1	1	12.10±0.12	30	0	0	0	0	13.79±0.18

Table 3. ANOVA for Full Quadratic Model

Sources	SS	DF	MS	F-value	Probe>F
Model	107.24	14	7.66	19.23	<0.0001
Residual	5.98	15	0.40		
Lack of fit	5.80	10	0.58	16.95	0.0030
Pure error	0.17	5	0.034		
Cor Total	113.22	29			

Std. Dev = 0.63; CV = 5.26%; R^2 = 0.9472; Adj R^2 = 0.8980; Adeq Precision = 16.980

Table 4. Significance Test of Regression Coefficient

Variables	Coefficient（standard error）	Computed *t* value	*P* value
Intercept	13.75(1)	68.20	<0.0001
X_1	-1.38(1)	115.39	<0.0001
X_2	-0.19(1)	2.15	0.1635
X_3	-0.36(1)	7.99	0.0128
X_4	0.029(1)	0.049	0.8276
X_1^2	0.14(0.12)	1.44	0.2493
X_2^2	1.25(0.12)	108.04	<0.0001
X_3^2	0.64(0.12)	28.02	<0.0001
X_4^2	0.16(0.12)	1.79	0.2011
$X_1 X_2$	0.035(0.16)	0.050	0.8268
$X_1 X_3$	0.055(0.16)	0.12	0.7334
$X_1 X_4$	0.62(0.16)	15.37	0.0014
$X_2 X_3$	0.32(0.16)	3.99	0.0643
$X_2 X_4$	0.096(0.16)	0.416605	0.5515
$X_3 X_4$	9.188E-03(0.16)	-0.9057	0.9543

The adjusted determination coefficient (Adj R^2 = 0.8980) was also satisfactory to confirm the significance of the model. And the "Pred R-Squared" of 0.7025 is in reasonable agreement with the "Adj R^2" of 0.8980. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Here the ratio of 16.980 indicates an adequate signal. A lower value of coefficient of variation (CV = 5.26%) showed that the experiments conducted were precise and reliable (Box *et al.* 1978; Zheng *et al.* 2008). This model can be used to navigate the design space.

The Student's t test and *P* values listed in Table 4 were used as a tool to check the significance of each coefficient, which also indicated the interaction strength between each independent variable. The larger the magnitude of *t*-test and smaller the P-value, the more significant is the corresponding coefficient (Lee and Wang 1997; Elibol 2004; Zheng *et al.* 2008). In this case, yeast extract had a strong negative linear effect on the response (P <<0.05), and $(NH_4)_2SO_4$ also showed a significant effect, whereas glucose and K_2HPO_4 were not significant in the range of this study. Significant interactions were noted between yeast extract and K_2HPO_4. The quadric effects of glucose and $(NH_4)_2SO_4$ were also significant. Yeast extract, as an organic nitrogen source, could promote the growth of cells effectively, but it decreased the carbon source flowing to the production of 2,3-BD. $(NH_4)_2SO_4$, as inorganic salt, was beneficial for the production of 2,3-BD with balance of the cell growth in suitable concentration. Glucose, as carbon source, is necessary for cell growth and production; however, if glucose concentration is too high, cell growth is restrained and the production is limited. On the other hand, if it is too low, the final concentration of the product would be low.

The results of validation experiments (Fig. 1) indicated that the optimal initial compositions of yeast extract, glucose, $(NH_4)_2SO_4$, and K_2HPO_4 were 2 g/L, 60 g/L, 6.23 g/L, and 11 g/L, respectively. Under this condition, 20.93 g/L of 2,3-BD concentration were obtained, which was coincident with the model prediction (19.81 g/L) by Design Expert, and the corresponding 2,3-BD yield was 0.38 g 2,3-BD/g consumed glucose.

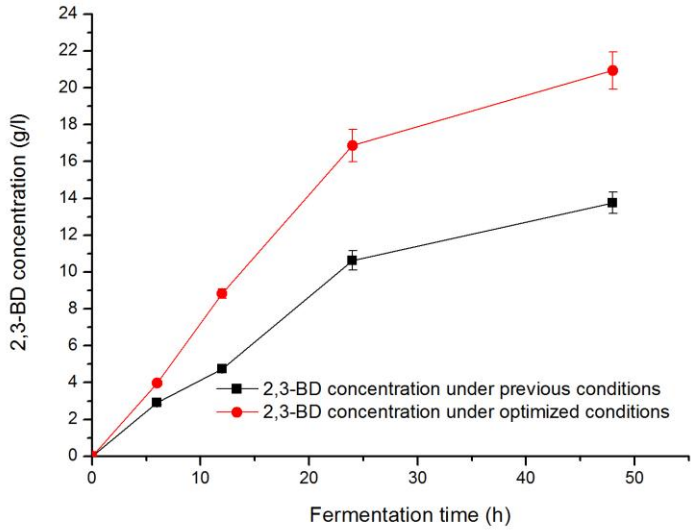

Fig. 1. Time courses of concentrations of 2,3-BD by *K. pneumoniae* CGMCC1.9131 under optimized and previous conditions

Furthermore, the interaction effects of different variables were analyzed. The 3D response surfaces shown in Fig. 2 are generally the graphical representation of the regression equation, in which each figure presented the effect of two variables on the production of 2,3-BD, while the other two variables were held at zero level. There were no significant interactions between different variables except for yeast extract and K_2HPO_4. With lower yeast extract concentration and higher K_2HPO_4, more 2,3-BD could be obtained in the fermentation, which was verified by the validation experiments.

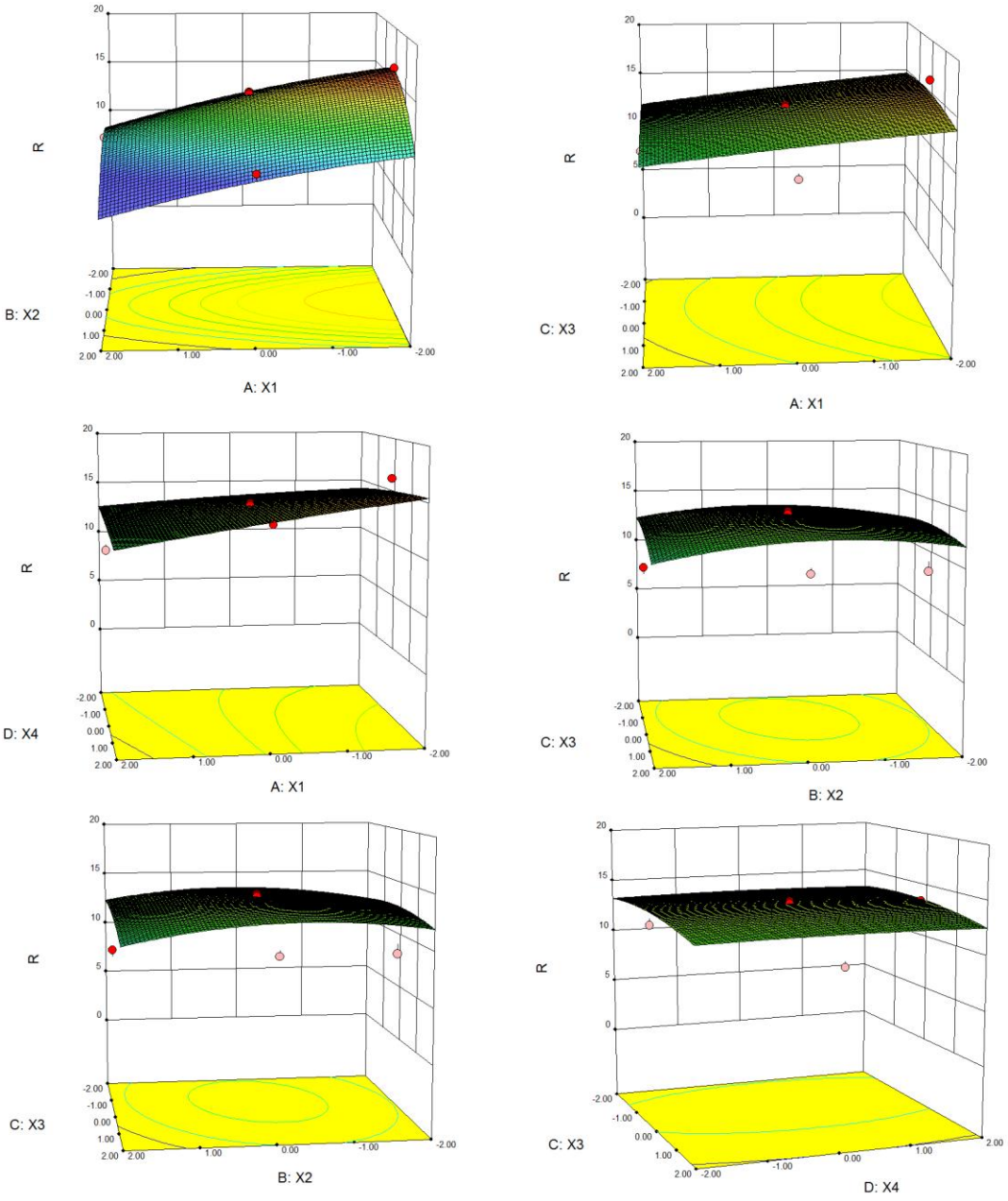

Fig. 2. Response surface figure of the mutual effects of different two variables on 2,3-BD production. R: 2,3-BD (g/l), X_1: Yeast extract concentration (g/L), X_2: Glucose concentration (g/L), X_3: $(NH_4)_2SO_4$ concentration (g/L), X_4: K_2HPO_4 concentration (g/L)

2,3-BD Production with Addition of Different Concentrations of EDTA Na_2

During the production of 2,3-BD it was found that there was still substrate left after 48 h fermentation in the optimal medium. To further improve the production of 2,3-BD and the utilization of substrate, EDTA Na_2, a commonly used metal chelating agent, was further added to the medium. EDTA Na_2 can promote the disintegration of Gram-negative bacteria's LPS (lipopolysaccharide) and render the peptidoglycan layer exposed, resulting in the improvement of cell permeability, which might increase the mass transfer during the fermentation (Loretta 1965). Five concentration levels were studied, and the experimental results are shown in Fig. 3. With addition of EDTA Na_2, the production of 2,3-BD was improved, and the substrate was consumed, but there was still more than 5 g/L glucose left when no EDTA Na_2 was added. However, the addition of EDTA Na_2 could restrain the growth of cells (Liu *et al.* 2010), so during the earlier stage of fermentation, the utilization rate of substrate and formation rate of 2,3-BD were relatively lower. As shown in Fig. 3, 0.5 g/L of EDTA Na_2 was found to be the most suitable for increasing 2,3-BD concentration to 26.20 g/L with corresponding 90% of theoretic yield. However, when no EDTA Na_2 was added, the 2,3-BD concentration was 20 g/L, which was only 76% of theoretic yield.

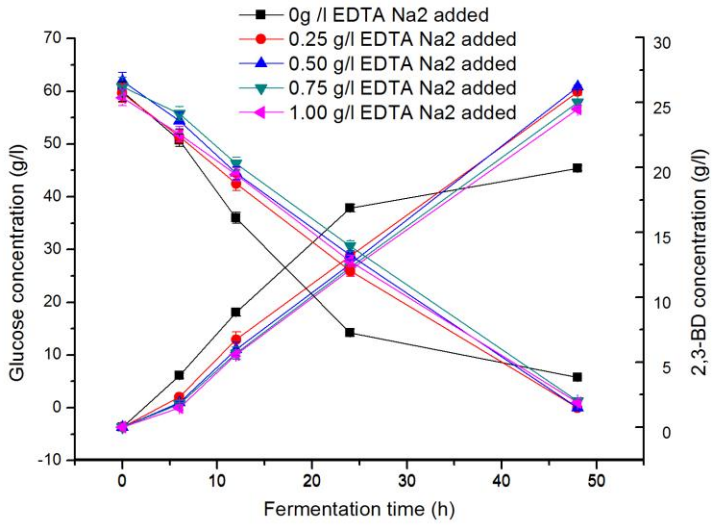

Fig. 3. Time courses of concentrations of 2,3-BD by *K. pneumoniae* CGMCC1.9131 in addition of different concentration of EDTA Na_2

2,3-BD Production with Enzymatic Hydrolyzate of Sugarcane Bagasse as Carbon Source

By different pretreatment of sugarcane bagasse, the concentrations of glucose and xylose were somewhat different in the enzymatic hydrolyzate (Zhao *et al.* 2011). The enzymatic hydrolyzate of alkali-PAA pretreated bagasse contained 30.54 g/L glucose and 13.87 g/L xylose after steam sterilization, while the enzymatic hydrolyzate of dilute acid–pretreated bagasse contained 42.59 g/L glucose and 5.36 g/L xylose. After addition of other nutrient compositions as above optimized concentrations, the hydrolyzates were used for 2,3-BD production as shown in Figs. 4 and 5. It can be known that glucose could be well converted to 2,3-BD in both hydrolyzates, and xylose also could be converted to

2,3-BD to some extent. However, there was still a large part of xylose left in the fermentation broth of alkali-PAA pretreated bagasse hydrolyzate, and the same result was shown in the production of ethanol from waste house hydrolyzate (Okuda *et al.* 2007). To examine the xylose uptake capability under simple conditions eliminating inhibitory factors, fermentation using only xylose as substrate was conducted. As listed in Table 5, *K. pneumoniae* CGMCC1.9131 consumed xylose for only 33.75% in the fermentation using 67.22 g/L xylose as a sole carbon source. However, as shown above, glucose could be completely consumed as a sole carbon source. So, the final concentration of 2,3-BD (17.35 g/L) for the dilute acid pretreated material was considerably higher than that (14.53 g/L) of the alkali-PAA sample. Nevertheless, the yield was 0.43 g 2,3-BD/g consumed sugar for enzymatic hydrolyzate of alkali-PAA sample, which is higher than that (0.36g 2,3-BD/g consumed sugar) of dilute acid-preated bagasse. Thus, the experimental results demonstrated that the enzymatic hydrolyzate of sugarcane bagasse could be well used for 2,3-BD production.

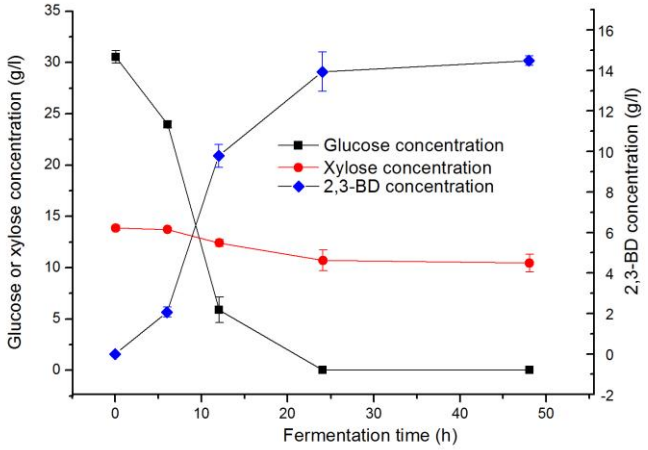

Fig. 4. Fermentation of glucose and xylose in enzymatic hydrolysis of sugarcane bagasse by alkali-PAA pretreatment to produce 2,3-BD

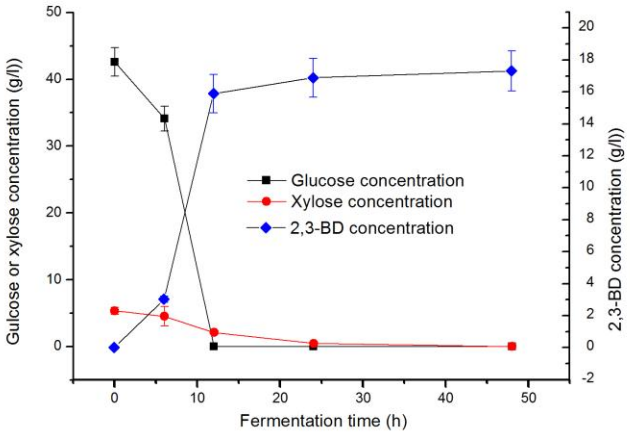

Fig. 5. Fermentation of glucose and xylose in enzymatic hydrolysis of sugarcane bagasse by dilute acid pretreatment to produce 2,3-BD

Table 5. Fermentation of 2,3-BD with Xylose as Substrate by *K. pneumoniae* CGMCC1.9131

Time	Concentration ($g \cdot L^{-1}$)		Xylose uptake ratio(%)	Cell dry weight ($g \cdot L^{-1}$)
	Xylose	2,3-Butanediol		
0	67.22±1.08	0.00	0.00	0.00
12	66.62±0.48	0.00	0.89	0.45±0.08
24	61.48±1.18	2.94±1.34	8.54	1.23±0.11
36	47.75±2.15	9.78±0.12	28.96	3.56±0.17
48	44.53±2.43	11.10±0.19	33.75	3.71±0.22

CONCLUSIONS

1. The fermentation conditions for 2,3-butanediol (2,3-BD) production by *Klebsiella pneumoniae* CGMCC1.9131 were optimized statistically in shake flasks with Response Surface Methodology (RSM). It was found that yeast extract as an organic nitrogen source could promote the growth of cells effectively, but it decreased the carbon source flowing to the production of 2,3-BD. $(NH4)_2SO_4$ as inorganic salt was beneficial for the production of 2,3-BD with the balance of the cell growth in suitable concentration. The optimal initial compositions of yeast extract, glucose, $(NH4)_2SO_4$, and K_2HPO_4 were 2 g/l, 60 g/l, 6.23 g/l and 11 g/l, respectively. Under this condition, 20.93 g/l of 2,3-BD with 0.38 g 2,3-BD/g consumed glucose were obtained.
2. Addition of EDTA Na_2 could improve the glucose to 2,3-BD conversion. 0.5 g/L of EDTA Na_2 was found to be the most suitable for increasing 2,3-BD concentration to 26.20 g/L with a corresponding 90% of theoretic yield. However, when no EDTA Na_2 was added, the 2,3-BD concentration was 20 g/L, which was only 76% of theoretic yield.
3. The enzymatic hydrolyzate of PAA-pretreated and dilute acid-pretreated sugarcane bagasse were further used as feedstock to produce 2,3-BD under the optimized conditions, and the yields of 2,3-BD were 0.36 and 0.43 g/g consumed sugars, respectively.

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