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Research Paper

The implementation of high fermentative 2,3-butanediol production from xylose by simultaneous additions of yeast extract, Na_2EDTA, and acetic acid

WangXiao-Xiong [1], HuHong-Ying [1,2], LiuDe-Hua [3] and SongYuan-Quan [3]

[1] Environmental Simulation and Pollution Control State Key Joint Laboratory, School of Environment, Tsinghua University, Beijing 100084, PR China
[2] State Environmental Protection Key Laboratory of Microorganism Application and Risk Control (SMARC), Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, PR China
[3] Department of Chemical Engineering, Tsinghua University, Beijing 100084, PR China

The effective use of xylose may significantly enhance the feasibility of using lignocellulosic hydrolysate to produce 2,3-butanediol (2,3-BD). Previous difficulties in 2,3-BD production include that the high-concentration xylose cannot be converted completely and the fermentation rate is slow. This study investigated the effects of yeast extract, ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), and acetic acid on 2,3-BD production from xylose. The central composite design approach was used to optimize the concentrations of these components. It was found that simultaneous addition of yeast extract, Na_2EDTA, and acetic acid could significantly improve 2,3-BD production. The optimal concentrations of yeast extract, Na_2EDTA, and acetic acid were 35.2, 1.2, and 4.5 g/L, respectively. The 2,3-BD concentration in the optimized medium reached 39.7 g/L after 48 hours of shake flask fermentation, the highest value ever reported in such a short period. The xylose utilization ratio and the 2,3-BD concentration increased to 99.0% and 42.7 g/L, respectively, after 48 hours of stirred batch fermentation. Furthermore, the 2,3-BD yield was 0.475 g/g, 95.0% of the theoretical maximum value. As the major components of lignocellulosic hydrolysate are glucose, xylose, and acetic acid, the results of this study indicate the possibility of directly using the hydrolysate to effectively produce 2,3-BD.

Introduction

Bio-refining using renewable biomass has recently attracted attention as an alternative means to provide sustainable fossil fuel resources [1]. 2,3-Butanediol (2,3-BD), an important industrial chemical and liquid fuel, can be produced through bio-refining using biomass such as hydrolyzed cellulose. 2,3-BD is a versatile platform chemical and is used by numerous consumers and manufacturers in the food, cosmetics, and medicine industries [2–5]. 2,3-BD can be dehydrated into 1,3-butandiene, commonly used to synthesize rubber and resins [6]. This chemical is a potential aviation fuel because of its high calorific value (27,198 J/g) [7,8]. As the most abundant and low-cost biomass on the earth [5], lignocellulose could possibly be used as a raw material to produce 2,3-BD. Behind glucose, xylose is the second-most concentrated sugar generated during lignocellulose hydrolysis [8]. Yan et al. [9] reported that the glucose-to-xylose sugar ratio in completely hydrolyzed corn stover solution is approximately 2:1 (w/w). The utilization ratio of lignocellulose is approximately 30% when only glucose is used [10]. This ratio increases to more than 50% when both glucose and xylose are used [11,12]. Hence, the investigation of using xylose as a carbon source to produce 2,3-BD is a necessary step to improve the utilization ratio of lignocellulose hydrolysis.

Klebsiella species, particularly *Klebsiella pneumoniae*, demonstrate satisfactory performance for the fermentative production of 2,3-BD [8]. Glucose can be used to produce 2,3-BD through *K. pneumoniae* fermentation [13–15]. However, highly concentrated xylose cannot be completely utilized to produce 2,3-BD using this system [15]. Furthermore, the rate of 2,3-BD production from

Corresponding author: Wang, X.-X. (lzsx1991@qq.com)

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xylose is lower than that from glucose, which is not ideal for industrial production. The fermentation time of shake flasks usually range from 72 to 96 hours [16,17]. The primary cause of the above problems is the shortage of several components in the medium. Thus, the use of *K. pneumoniae* for the fermentative production of 2,3-BD with high xylose utilization ratio, 2,3-BD yield, and fermentation rate is worthy of investigation.

Yeast extract is a commonly used organic nitrogen source for the fermentative production of 2,3-BD. This component significantly promotes *K. pneumoniae* growth and 2,3-BD accumulation when xylose is used as a substrate [18]. When producing 2,3-BD from glucose, adding ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) can improve the glucose utilization of *K. pneumoniae*. Adding 0.5 g/L Na_2EDTA may increase the yield of 2,3-BD from 76 to 90% [15]. A link reportedly exists between the 2,3-BD formation pathway and acidification. The optimum concentration of acetic acid in the glucose production of 2,3-BD is 1 g/L [19]. To the best of our knowledge, the effects of adding Na_2EDTA and acetic acid on xylose fermentation during 2,3-BD production have not been previously studied.

This study aims to investigate the effects of simultaneously adding yeast extract, Na_2EDTA, and acetic acid during fermentative production of 2,3-BD with xylose as a sole substrate. The added concentrations of these components were optimized to completely utilize xylose and expedite fermentation. Optimization yielded the highest 2,3-BD production and xylose transformation ratio ever reported.

Materials and methods

Microorganism

The strain used to produce 2,3-BD was *K. pneumoniae* HR521 LDH (CGMCC 1.9131), previously isolated from soil samples [20] by Song et al. [21]. The D-(−)-lactic acid biosynthesis pathway gene, ldhA, of this strain was removed to prevent lactic acid production during fermentation and thus increase the yield of the desired product. Earlier studies showed that the stereoisomer of 2,3-BD produced by the strain was optically inactive meso-form (86–95%) and levo- [L-(+)-] form (5–14%). The strain is currently stored in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China).

Medium and culture conditions

K. pneumoniae HR521 LDH was maintained on agar slants composed of 20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 20 g/L agar at pH 7.0. *K. pneumoniae* HR521 LDH was incubated on the slants, cultivated at 37°C for 12 hours, and then stored at 4°C [12].

In seed preparation, a full loop of *K. pneumoniae* HR521 LDH from a freshly prepared slant was inoculated into 250-mL shake flasks containing 100 mL of fresh seed medium composed of 20 g/L xylose, 10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl at pH 7.0. The medium was then incubated on a rotary shaker at 200 rpm for 12 hours at 37°C.

The optimum concentration of the xylose substrate for 2,3-BD production was reported as 90 g/L [16,17]. Thus, 90 g/L xylose was used as the initial substrate concentration in the present study. The other components within the medium were as follows: 2 g/L $(NH_4)_2SO_4$, 7 g/L K_2HPO_4, 10 g/L $KH_2PO_4{\cdot}3H_2O$, 4 g/L sodium citrate, 0.1 g/L $MgSO_4$, 0.05 g/L $CaCl_2$, 0.01 g/L $ZnSO_4{\cdot}7H_2O$, 0.005 g/L $FeSO_4{\cdot}7H_2O$, and 0.005 g/L $MnSO_4{\cdot}7H_2O$ at pH 6.5. A 2% (v/v) seed culture was inoculated into 250-mL shake flasks with a working volume of 100 mL or 5% (v/v) inoculated into a 5-L, stirred fermenter (BIOSTAT-B Plus, B. Braun Co., Germany) with a working volume of 4 L. The cultivation was performed at 37°C and 200 rpm in the shake flasks and at 37°C and 300 rpm with an airflow rate of 2.5 vvm in the stirred fermenter.

TABLE 1

Levels of variables used in CCD experimental design

Variables (g/L)		Levels				
		−1.41	−1	0	+1	+1.41
A	Yeast extract	3.79	10.0	25.0	40.0	46.2
B	Na_2EDTA	0.10	0.60	1.80	3.00	3.50

Analytical methods

The concentration of *K. pneumoniae* HR521 LDH was determined *via* an optical density calibration curve determined at 600 nm (OD_{600}) using a spectrophotometer (722E Spectrophotometer, Shanghai Analytical Instrument Factory, China). The dry cell weight was calculated from the optical density using the calibration curve of the strain.

The concentrations of xylose, glucose, 2,3-BD, acetic acid, succinic acid, and ethanol were determined using a high-performance liquid chromatography (HPLC) system (10 AVP HPLC, Shimadzu Co., Japan) with an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad, Palo Alto, CA) at 65°C equipped with a RID-10A refractive index detector. The mobile phase was 0.005 mol/L H_2SO_4 solution at 0.8 mL/min.

Statistical experimental design

Response surface methodology (RSM) has been used to determine the optimum levels of a medium because of its high accuracy and efficiency [15]. In the present experiments, the central composite design (CCD) was employed to optimize the initial concentrations of yeast extract and Na_2EDTA. The CCD is an efficient RSM for medium component optimization [15]. The experimental plan was designed using Design-Expert V 8.0.6.1 software (Stat-Ease Inc., USA). The two independent factors were studied at five levels (−1.41, −1, 0, +1, and +1.41), and a set of 13 experiments was performed (Table 2). Table 1 illustrates the levels of each variable included in the experimental design.

The factors were coded according to the following equation (Eqn 1) [1]:

$$X_i = \frac{x_i - x_0}{\Delta x_i} \quad i = 1, 2, \ldots, k \tag{1}$$

where X_i is the coded independent factor, x_i is the real independent factor, x_0 is the value of x_i at the center point, and Δx_i is the step change value. The behavior of the system can be explained by the following second-order polynomial equation (Eqn 2):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad i, j = 1, 2, \ldots, k \tag{2}$$

where Y is the predicted response (i.e., 2,3-BD concentration in g/L), X_i and X_j are the coded independent factors, β_0 is the intercept, β_i is