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Enhanced 2,3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy

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ABSTRACT

Batch fermentative production of 2,3-butanediol by *Klebsiella oxytoca* was investigated using various oxygen supply methods though varying agitation speed. Based on the analysis of three kinetic parameters including specific cell growth rate (μ), specific glucose consumption rate (q_s) and specific 2,3-butanediol formation rate (q_p), a two-stage agitation speed control strategy, aimed at achieving high concentration, high yield and high productivity of 2,3-butanediol, was proposed. At the first 15 h, agitation speed was controlled at 300 rpm to obtain high μ for cell growth, subsequently agitation speed was controlled at 200 rpm to maintain high q_p for high 2,3-butanediol accumulation. Finally, the maximum concentration of 2,3-butanediol reached 95.5 g l^{-1} with the yield of 0.478 g g^{-1} and the productivity of 1.71 g l^{-1} h^{-1}, which were 6.23%, 6.22% and 22.14% over the best results controlled by constant agitation speeds.

1. Introduction

Development of bio-refineries has recently attracted increasing attention as a means to provide sustainable alternative solutions to depleting petroleum resources and environmental pollution. Many chemicals, which could only be produced by chemical processes in the past, could potentially be generated biologically from annually renewable resources (Ragauskas et al., 2006). Microbial production of 2,3-butanediol (2,3-BD) is one of the examples. Interest in this bioprocess has been increasing recently due to that 2,3-BD has large number of industrial applications and this course would alleviate the dependence on oil supply for the production of platform chemicals. The dehydration of 2,3-BD yields the industrial solvent methyl ethyl ketone (Tran and Chambers, 1987). Further dehydration produces 1,3-butadiene, which is the building block of synthetic rubber (van Haveren et al., 2007). And the high octane rating of 2,3-BD makes it a potential aviation fuel (Magee and Kosaric, 1987; Wu et al., 2008). Besides, 2,3-BD has potential applications in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods and pharmaceuticals (Garg and Jain, 1995; Syu, 2001).

2,3-BD could be produced from carbohydrates by enteric bacteria of the genera *Enterobacter* and *Klebsiella* and by bacilli such as *Paenibacillus polymyxa* via the mixed acid–butanediol fermentation pathway (Kosaric et al., 1992; Syu, 2001). In the previous study, it was found that the oxygen supply was a critical factor for high level production of 2,3-BD. Jansen et al. (1984) found that high oxygen supply favored cell mass formation at the expense of 2,3-BD production. While decreasing the oxygen supply would increase 2,3-BD yield, but it would decrease the overall conversion rate due to the lower cell concentration formed (Sablayrolles and Goma, 1984). This indicated that the oxygen supply condition for improving reactor productivity was different from that for maximizing 2,3-BD yield and final 2,3-BD concentration. The above information demonstrated the positive or negative effect of the oxygen supply on 2,3-BD production. Therefore, it was necessary to set up a proper oxygen supply strategy to ensure efficient 2,3-BD production with high concentration, high yield and high productivity. In the previous study, oxygen transfer rate (OTR), oxygen transfer coefficient (k_La) and respiratory quotient (RQ) guided oxygen supply strategies were applied in 2,3-BD fermentation successfully (Beronio and Tsao, 1993; Fages et al., 1986; Zeng et al., 1994). However, the parameters of OTR, k_La and RQ were not so easy to control, thus restricting the application of those strategies.

In the present study, a simple oxygen supply method based on agitation speed control was set up for efficient 2,3-BD fermentation. The processes of 2,3-BD fermentation by *Klebsiella oxytoca* were compared at different oxygen supply conditions by changing agitation speed. Subsequently, a two-stage agitation speed control strategy, aimed at achieving high concentration, high yield and high productivity of 2,3-BD, was designed based on the kinetic analysis of batch processes controlled by single-agitation speed and was confirmed experimentally.

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2. Methods

2.1. Microorganism and media

The strain for 2,3-BD production used in this work was a low-acid producing *K. oxytoca* ME-UD-3 with acetoin and ethanol as the main byproducts during 2,3-BD production (Ji et al., 2008). The culture was maintained on Luria–Bertani (LB) agar slant at 4 °C. The seed medium was composed of (g l^{-1}): peptone, 10; beef extract, 3; NaCl, 5 and pH 6.5. The fermentation medium was (g l^{-1}): glucose, 200; K_2HPO_4, 13.7; KH_2PO_4, 2.0; $(NH_4)_2HPO_4$, 3.3; $(NH_4)_2SO_4$, 6.6; $MgSO_4 \cdot 7H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.001; $CaCl_2 \cdot 2H_2O$, 0.01; EDTA, 0.05 and pH 6.5.

2.2. Culture methods

For seed preparation, *K. oxytoca* ME-UD-3 from a fresh slant tube was inoculated into 100 ml fresh seed medium in 250-ml flasks and cultivated on a rotary shaker at 200 rpm for 24 h. Seed culture (5%, v/v) was then inoculated into the fermentation medium. Batch fermentation was carried out in a 3-l stirred fermenter (BioFlo 100; New Brunswick Scientific Co., NJ, USA) with a working volume of 2 l. All cultivation was carried out at 37 °C, and pH was controlled at 6.5 automatically by adding 3 M NaOH. The aeration rate was controlled at 1.0 volume of air per volume of liquid per minute (vvm) for all the experiments, while the agitation speed was controlled at 100, 200, 300 and 400 rpm in the batch fermentation, respectively. Under the jar-fermentation condition, the oxygen transfer coefficient k_La under each agitation speed was measured as 4.76 h^{-1}, 18.1 h^{-1}, 26.7 h^{-1}, 46.9 h^{-1}, respectively, by the method of using Na_2SO_3 solution. The dissolved oxygen concentrations under different operation condition were expressed in the term of dissolved oxygen saturation level (DOC, %), while 100% dissolved oxygen saturation level corresponded to an actual dissolved oxygen concentration of about 8.2 mg l^{-1} at 37 °C, 1.0 atm. These operation conditions were adopted in different batch fermentation experiments, to investigate the effects of different agitation speeds on cell growth, 2,3-BD production and glucose consumption.

2.3. Analytical methods

Dry cell weight (DCW, g l^{-1}) was determined from a calibration curve of known DCW and the corresponding optical density at 600 nm (OD_{600}) (DCW = 0.4892 × OD_{600} + 1.2437). OD_{600} of the broth was measured using a UV–visible spectroscopy system (DU-640, Beckman, USA) with appropriate dilution. The DCW calibration curve was determined from the weight difference created by filtering and washing known volumes of broth through predried filter paper followed by drying at 100 °C for 24 h. This calibration had a linear regression coefficient of 98% over the optical density range 0.1–0.9. Glucose, 2,3-BD and the two byproducts (acetoin, ethanol) were measured by high-performance liquid chromatography (Summit P 680 HPLC, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; Aminex HPX-87 H Ion Exclusion Column 300 mm × 7.8 mm, Bio-Rad, USA) under the following conditions: sample volume 10 μl; mobile phase 0.005 M H_2SO_4; flow rate 0.2 ml min^{-1}; and column temperature 60 °C.

2.4. Kinetic parameters calculation

The specific cell growth rate (μ, h^{-1}), specific glucose consumption rate (q_s, h^{-1}) and specific 2,3-BD formation rate (q_p, h^{-1}) were estimated from experimental or fitted data of cell growth (x, g l^{-1}), residual glucose concentration (s, g l^{-1}), and 2,3-BD production (p, g l^{-1}) by Eqs. (1)–(3), respectively (Mao and Zhong, 2004; Zheng et al., 2002). The fitted data were obtained by interposing between experimental data of cell growth, residual glucose concentration or 2,3-BD production at definite time (dt = 0.1 h) with the approximation method of cubic spline interpolation in Origin software (Version 7.5, OriginLab Corp., Northampton, MA, USA).

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta x}{\Delta t} \quad (1)$$

$$q_s = -\frac{1}{x}\frac{ds}{dt} = -\frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta s}{\Delta t} \quad (2)$$

$$q_p = \frac{1}{x}\frac{dp}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta p}{\Delta t} \quad (3)$$

3. Results

3.1. Time course of 2,3-BD fermentation at different agitation speeds

Effects of agitation speed (100, 200, 300 and 400 rpm) on 2,3-BD fermentation were investigated, respectively. The results indicated that agitation speed played a vital role in 2,3-BD production. As shown in Fig. 1A–D, the relatively higher final 2,3-BD concentrations up to 89.9 g l^{-1} and 86.2 g l^{-1} were obtained at the agitation speeds of 200 rpm and 300 rpm, respectively. While when the agitation speed was higher or lower, the final 2,3-BD concentration decreased (78.5 g l^{-1} at 400 rpm, 79.4 g l^{-1} at 100 rpm). This indicated that either lower agitation speed (100 rpm) or higher agitation speed (400 rpm) was not beneficial for 2,3-BD production. The reason might be that ethanol and acetoin were relatively highly accumulated as the main byproduct, respectively, in these two cases (Fig. 1A and D). Furthermore, although the final 2,3-BD concentration at the agitation speed of 200 rpm was higher compared with the agitation speed of 300 rpm, the former 2,3-BD productivity was lower than the latter (1.40 g l^{-1} h^{-1} at 200 rpm, 1.44 g l^{-1} h^{-1} at 300 rpm). The observations described above indicated that the optimum agitation speed for obtaining high concentration and high yield of 2,3-BD was not suitable for ensuring high 2,3-BD productivity. It can therefore be concluded that high concentration, high yield and high productivity of 2,3-BD could not be achieved simultaneously by controlling a constant agitation speed throughout the whole culture process.

3.2. Kinetic analysis of 2,3-BD fermentation at different agitation speeds

To analyze the kinetic characteristics of the above two processes at the agitation speed of 200 rpm and 300 rpm, three kinetic parameters, including μ, q_s and q_p, were calculated based on the data of Fig. 1B and C. As shown in Fig. 2, μ, q_s and q_p had similar tendencies with the maximum value appeared at about 5 h. Comparing with the agitation speed of 200 rpm, μ and q_s were higher at the agitation speed of 300 rpm at the beginning of 2,3-BD fermentation (about 15 h). It showed that the agitation speed of 300 rpm was better for cell growth and glucose consumption during the first 15 h. But after 15 h the agitation speed of 200 rpm was beneficial for 2,3-BD formation with a high valve of q_p. Combining this result with that obtained in the experiments of constant agitation speed fermentation, it could be concluded that the lower agitation speed was beneficial for high 2,3-BD accumulation in the later stage of cultivation. Based on the analysis of μ, q_s and q_p, a two-stage agitation speed control strategy was therefore proposed. In this strategy, the agitation speed was controlled at 300 rpm in the first 15 h to maintain high μ and q_s for fast cell growth and