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An Effective and Simplified Fed-Batch Strategy for Improved 2,3-Butanediol Production by *Klebsiella oxytoca*

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Abstract Substrate concentration in 2,3-butanediol (2,3-BD) fermentation could not be controlled well in traditional feeding strategies, such as constant, impulse, and exponential feeding strategies. In the present study, fermentative 2,3-BD production by *Klebsiella oxytoca* was investigated under different batch and fed-batch strategies. The glucose-feedback fed-batch strategy was proved to be not effective for economical 2,3-BD production for the inability of timely feeding, leading that the bacteria reused 2,3-BD as carbon source for cell growth. Based on the phenomena that the byproducing acids caused the pH declining and the requirement of maintaining the pH at a proper level for both cell growth and 2,3-BD accumulation, an improved strategy of pH-stat fed-batch culture with glucose and sodium hydrate fed at the same time was established. Thus, the residual glucose concentration could be controlled through the adjustment of pH automatically. At last, efficient 2,3-BD production was fulfilled under this fed-batch strategy, and the highest 2,3-BD concentration, productivity, and yield were 127.9 g/l, 1.78 g/(l•h), and 0.48 g/g (2,3-BD/glucose), respectively, compared to 98.5 g/l, 1.37 g/(l•h), and 0.43 g/g obtained in glucose-feedback fed-batch strategy. This feeding strategy was simple and easy to operate and could be feasible for industrial 2,3-BD production in the future.

Keywords 2,3-Butanediol · *Klebsiella oxytoca* · pH-stat · Fed-batch

Introduction

Recently, bio-refineries has 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 synthesized by microorganism using renewable bioresources [1, 2]. One of

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the examples is microbial production of 2,3-butanediol (2,3-BD), which is a kind of promising bulk chemical having broad industrial applications. The dehydration of 2,3-BD yields the industrial solvent methyl ethyl ketone [3]. Further dehydration produces 1,3-butadiene, which is the building block of synthetic rubber [4]. And the high octane rating of 2,3-BD makes it a potential aviation fuel [5, 6]. Besides, 2,3-BD has potential applications in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals [7, 8].

2,3-BD accumulation had been widely identified in different bacteria including species of *Klebsiella* and *Paenibacillus*, as well as some species of *Enterobacter*, *Serratia*, and *Aeromonas* [8, 9]. Among all these strains, *Klebsiella* species were often used for 2,3-BD production for their broad substrate spectrum and cultural adaptability [10]. In *Klebsiella* species, the biosynthesis of 2,3-BD was via a mixed acid-2,3-butanediol fermentation process [9], the accompanying formed acids such as lactic and acetic acid could cause the culture pH decreasing during the fermentation process, and then cell growth was inhibited [11]. And one of the key enzymes involved in 2,3-BD production, i.e., α-acetolactate synthase (EC 4.1.3.18, also called pH 6.0 α-acetolactate-forming enzyme), was sensitive to pH value and could not function well above or under pH 6.0 [12]. Therefore, it is important to control the pH value at a proper level for both cell growth and 2,3-BD production.

In the previous microbial 2,3-BD production process, different operation methods such as batch, fed-batch, and continuous fermentation strategies had been studied [13]. However, in the batch fermentation, the final 2,3-BD concentration and productivity were relatively low and cell growth was generally inhibited by the initial high substrate concentration [14]. Continuous fermentation could achieve a high productivity by supplying constant and usually stable growth rates and providing an essentially invariant microbial environment, but the 2,3-BD yield was relatively low [15]. Fed-batch culture, which was generally superior to the batch and continuous fermentation process, could avoid the effect of substrate inhibition by keeping the substrate concentration at a relatively low level [16, 17]. Reimann and Biebl [18] proposed a feeding method based on pH in which the carbon source and alkali were mixed together in 1,3-propanediol fed-batch fermentation. By this way, a continuous, automated substrate addition was achieved responding directly to the needs of the culture [18]. In this paper, a pH feedback-controlled feeding method was introduced to control the glucose concentration in 2,3-BD fermentation, and the advantage of this method was investigated to increase 2,3-BD production.

The aim of the present study was to develop a simple and effective fed-batch method for efficient biological 2,3-BD production [19, 20]. 2,3-BD fermentation was investigated under different batch and fed-batch strategies. And based on the phenomena that the byproducing acids caused the pH declining and the requirement of maintaining the substrate concentration at a proper level for both cell growth and 2,3-BD fermentation, an improved strategy of pH-stat fed-batch culture with glucose–sodium hydrate mixture was established. Thus, the residual glucose concentration could be controlled through the adjustment of pH automatically. At last, efficient 2,3-BD production was fulfilled.

Materials and Methods

Microorganism and Media

Klebsiella oxytoca ME-UD-3, a mutant from *K. oxytoca* CCTCC M207023 (China Center for Type Culture Collection), was used in this study for 2,3-BD production [21]. Luria–

Bertani medium was used as the seed medium. And the seed culture was prepared in a 250-ml Erlenmeyer flask containing 50 ml fresh seed medium inoculated with a full loop of *K. oxytoca* from fresh slant tube and cultivated at 37 °C on a rotary shaker at 200 rpm for 24 h and subsequently inoculated into the bioreactor at 5% (*v*/*v*). The initial fermentation medium was consisted of (g/l): glucose, 80 (fed-batch fermentation) or 220 (batch fermentation); 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; and pH 7.0 [22]. The glucose concentration in feeding medium was 800 g/l.

Batch Culture Methods

Batch fermentations were carried out at 3-l stirred fermenter (BioFlo 110, New Brunswick Scientific, USA) with a working volume of 2 l. The seed culture prepared previously was inoculated (5%, *v*/*v*) into the fermentation medium. The cultivation was carried out at 37 °C with the aeration rate and agitation speed at 1.0 vvm and 200 rpm, respectively, and pH was controlled at 6.0 automatically by adding 3 M NaOH.

Fed-Batch Culture Methods

Fed-batch fermentations were carried out at 3-l stirred fermenter with 1 l initial medium. All fed-batch fermentations were conducted with an initial glucose concentration of 80 g/l, and the feeding substrate was fed into the fermenter using a computer-coupled peristaltic pump, the other cultivation conditions were the same as the batch experiments. As the pH limits were set at 5.97 and 6.03. In the first phase, pH was not controlled, but when the culture pH dropped to the lower limit, pH controller would be activated, and it was maintained at 6.0 by automatic addition of 3 M sodium hydrate using a computer-controlled peristaltic pump. Glucose was fed according the needs of different control methods (Fig. 1).

Analytical Methods

The biomass concentration was expressed as dry cell weight (DCW), which was determined by measuring the optical density of broth at 600 nm using a UV-visible spectroscopy system (Lambda-25, Perkin-Elmer, USA). And one unit of optical density was determined to be equivalent to 0.35 g DCW per liter. Glucose was determined by a biosensor equipped with glucose oxidase electrode (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China). 2,3-BD, acetoin, acetic acid, lactic acid, and ethanol concentrations were determined via high performance liquid chromatography as indicated in our previous study [23].

Results and Discussion

pH-Stat Batch Culture

In the previous work, the strain was acclimated to be able to tolerant to initial glucose concentration as high as 300 g/l. So it was possible to achieve high 2,3-BD yields from high concentrations of glucose. Therefore, a pH-stat batch culture with high initial glucose concentration was investigated. Figure 2 showed the changes of the main parameters

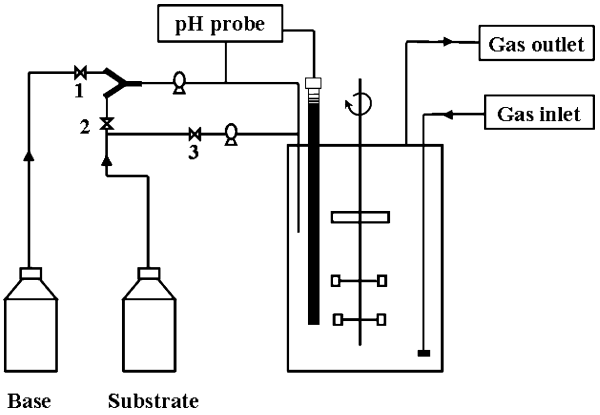

Fig. 1 The pH-stat batch and fed-batch culture system. pH-stat batch culture: Sodium hydrate was fed at a separate line to maintain the culture pH at 6.0. The valve 1 was opened and valves 2 and 3 were closed. pH-stat fed-batch culture with glucose feeding according to the residual glucose: Sodium hydrate was fed at a separate line to maintain the culture pH at 6.0. The valves 1 and 3 were opened and valve 2 was closed. pH-stat fed-batch culture with glucose and sodium hydrate feeding at the same time: The pH-stat fed-batch operation was activated when the culture pH dropped to the lower pH limit. The culture pH was maintained at 6.0 by feeding glucose and sodium hydrate at the same time. The valves 1 and 2 were opened and valve 3 was closed

(DCW, 2,3-BD, residual glucose, acetoin, acetic acid) during the course of pH-stat batch culture. After the first 8 h adaptation period, the cells entered the period of maximum growth until the end of fermentation. The glucose consumption rate was faster when the cell was in rapid growth, meanwhile accelerated 2,3-BD formation rate was observed with the glucose consumption. With the progress of fermentation, the 2,3-BD concentration increased and reached a relative steady level after 81 h. The productivity, maximum concentration, and yield of 2,3-BD were 1.06 g/(l•h), 86.2 g/l, and 0.39 g/g (2,3-BD/glucose), respectively.

The above batch culture was easy to operate. However, the productivity, concentration, and yield of 2,3-BD were relatively low. The batch culture needed a long adaptation period because cell growth was more or less inhibited by the initial high substrate concentration, and this would prolong the fermentation time thus lower the productivity. In batch culture, the bacteria were in vigorous growth after the adaptation period, and this would consume

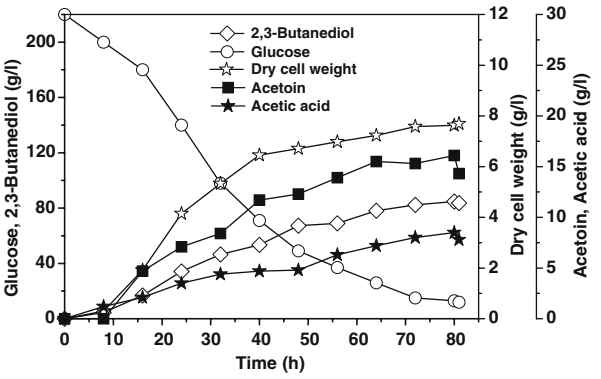

Fig. 2 Time course of 2,3-butanediol fermentation by *Klebsiella oxytoca* using pH-stat batch culture strategy

large parts of glucose to maintain cell growth. This indicated that it was necessary to maintain normal cell growth and metabolism, meanwhile minimize the amount of glucose consumed for cell growth to achieve higher 2,3-BD concentration. And a proper feeding strategy could be designed to resolve the above problem.

pH-Stat Fed-Batch Culture with Glucose Feeding According to the Residual Glucose

In the above strategy, the glucose concentration was too high in the prophase and inadequate in the anaphase, leading to the relatively low conversion efficiency. To solve this problem, a modified strategy of pH-stat fed-batch culture with glucose-feedback to maintain the residual glucose at a proper level, was therefore proposed. Samples were collected periodically to determine the residual glucose concentration, and then the glucose feeding rate was adjusted to keep the glucose concentration at a comparatively low level.

As shown in Fig. 3, during the first 8 h, the glucose concentration dropped from 80 to 30 g/l, during which the DCW increased rapidly. At the same time, 2,3-BD began to accumulate. After 8 h, glucose was added at different constant rate at each interval. The final 2,3-BD concentration was up to 98.5 g/l at 72 h with a yield of 0.43 g/g and the productivity of 1.37 g/(l•h). Compared with the pH-stat batch culture, the 2,3-BD concentration, yield, and productivity were significantly improved.

However, the feeding occasion was not so easy to determine due to the lack of glucose online analysis equipment, and this system was not sufficiently robust to control glucose concentrations consistently at the desired levels. Also, the feeding amount of glucose was according to the glucose consumption before interval, and thus could not to respond the real information. In some cases, this would cause the glucose not to be timely fed and 2,3-BD would be reused as the carbon source for cell growth [24]. Therefore, designing a proper feeding strategy which could resolve the problem and keep the glucose concentration at a relative low level would be a straightforward way for efficient 2,3-BD production.

pH-Stat Fed-Batch Culture with Glucose and Sodium Hydrate Feeding at the Same Time

In order to avoid the necessary procedure of determining suitable feeding occasion in the above strategy, a further modified pH-stat fed-batch culture strategy was established. Since the synthesis of 2,3-BD was a mixed acid fermentation process, the byproducing acids would lead the culture pH declining. So alkali such as sodium hydrate should be pumped to the medium to maintain the culture pH. Therefore, a pH-stat fed-batch culture with glucose

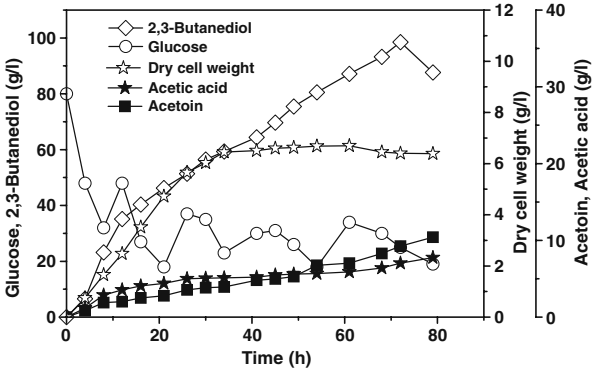

Fig. 3 Time course of 2,3-butanediol fermentation by *Klebsiella oxytoca* using pH-stat fed-batch culture strategy with glucose feeding according to the residual glucose

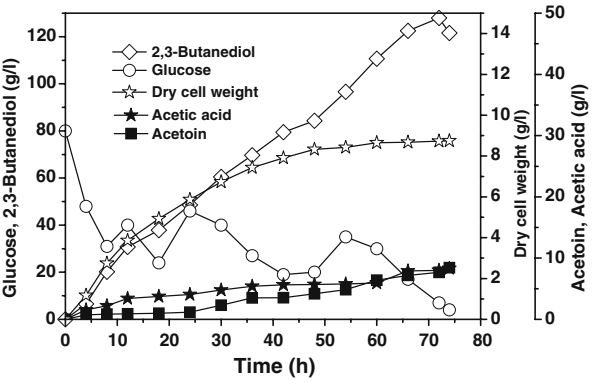

Fig. 4 Time course of 2,3-butanediol fermentation by *Klebsiella oxytoca* using pH-stat fed-batch culture strategy with glucose and sodium hydrate feeding at the same time

and sodium hydrate mixture was thus established, namely, a glucose reservoir and the base container were connected in parallel by using a double peristaltic head pump controlled by the computer system (Fig. 1). Glucose was fed with the sodium hydrate pumped into the culture to maintain pH by the base pump equipped in the fermentor. In this course, during the first phase of culture, pH was not controlled. The pH-stat fed-batch operation was activated when the culture pH dropped to the lower limit. And the culture pH was maintained at 6.0 by feeding the glucose–sodium hydrate mixture. Thus, glucose and sodium hydrate were fed at the same time. In practical operation process, the proportion of the glucose and sodium hydrate could be adjusted by regulating the velocity ratio of the two valves.

As shown in Fig. 4, in the first 8 h, pH was not controlled, the concentration of glucose swiftly dropped from 80 to 30 g/l. During this period, the DCW increased rapidly, while the yield of 2,3-BD was low. As time goes on, acetic acid was produced and thus, leading the culture pH drop to 6.0. Fed-batch operation was activated, thus, sodium hydrate was added to the culture to maintain pH and glucose was also fed into the culture at the same time. From 8 h to the end of fermentation, the glucose concentration was kept at 20–40 g/l which was beneficial for both cell growth and 2,3-BD production.

In this way, the glucose could be fed automatically according the production rate of byproducing acids. The strategy can realize continuous, fully automated fermentation

Table 1 The comparison of parameters at different batch and fed-batch culture strategies

Parameters	Method 1[a]	Method 2[b]	Method 3[c]
Fermentation time (h)	81	72	72
2,3-Butanedion (g/l)	86.2	98.5	127.9
Acetoin (g/l)	15.4	10.4	8.4
Acetic acid (g/l)	8.5	7.7	8.4
Residual glucose (g/l)	12	25	7
2,3-Butanediol yield (g/g)	0.39	0.43	0.48
2,3-Butanediol productivity (g/(l•h))	1.06	1.37	1.78

[a] pH-stat batch culture

[b] pH-stat fed-batch culture with glucose feeding according to the residual glucose

[c] pH-stat fed-batch culture with glucose and sodium hydrate feeding at the same time

process. The concentration of the inhibitory substrate glucose in the fermentor was kept at a relatively low but non-limiting level throughout the fermentation and pH could be controlled at 6.0. In the fermentation process, the substrate concentration in the fermentor and the substrate consumption rate could be estimated from the amount of added sodium hydrate and substrate. The method can be applied for the fermentation which exist a constant relationship between substrate consumption and acid formation. At last, efficient 2,3-BD production was fulfilled under this fed-batch strategy, and the highest 2,3-BD concentration was up to 127.9 g/l with a productivity of 1.78 g/(l•h).

Conclusions

In this paper, three different culture methods for fermentative 2,3-BD production were compared. An effective and simplified fed-batch culture strategy coupled with pH control was developed. At last, the maximum concentration of 2,3-BD reached 127.9 g/l with the byproducts and residual glucose under low concentrations (Table 1). The proposed strategy was simple and easy to operate and proved to be an effective method for efficient 2,3-BD production. Also, it would be feasible for industrial 2,3-BD production employed in the future and could be applied for other similar fermentation processes.

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