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Efficient 1,3-propanediol Production by Fed-Batch Culture of *Klebsiella Pneumoniae*: The Role of pH Fluctuation

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Abstract The fermentative production of 1,3-propanediol (1,3-PD) by *Klebsiella pneumoniae* under different fed-batch strategies was investigated. pH-stat fed-batch strategies proved to be not effective for economical 1,3-PD production for the existence of relatively high concentration of byproducts and residual glycerol at the end of the fermentation. However, in the pH-stat fed-batch strategy, an important phenomenon was observed that the yields of two main byproducts, 2,3-butanediol and lactic acid, were closely related to pH value. The dominant byproduct was 2,3-butanediol at a pH value of 5.0 to 6.5 but changed to be lactic acid at a pH value of 7.1 to 8.0. Based on the analysis of the phenomenon, a self-protection mechanism in *K. pneumoniae*, namely that the growing *K. pneumoniae* cells switch the metabolic pathways responding to environmental pH changes, was proposed. Thus a kind of feeding strategy was further applied during which the pH value was fluctuated between 6.3 and 7.3 periodically by feeding glycerol–ammonia mixture and sulphuric acid to make the metabolic pathways of 2,3-butanediol and lactic acid sub-active under the periodical low or high pH stress. At last, efficient 1,3-PD production was fulfilled under this fed-batch strategy, and the best results were achieved leading to 70 g/l 1,3-PD with a yield of 0.70 mol/mol glycerol and productivity of 0.97 g/l/h, while the two main byproducts and residual glycerol were under low concentrations.

Keywords 1,3-propanediol · *Klebsiella pneumoniae* · Glycerol · Fed-batch · pH fluctuation

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

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past, could potentially be generated biologically from annually renewable resources [1, 2]. Among them, 1,3-propanediol (1,3-PD) is a bulk chemical for the production of various high value-added derivatives. The applications of 1,3-PD and its derivatives include polymers, cosmetics, foods, lubricants, and medicines, in particular, 1,3-PD can be used as a monomer to synthesize a new type of polyester, polytrimethylene terephthalate (PTT) [3].

As the substrate of 1,3-PD fermentation, glycerol is inexpensive and renewable, which can be obtained by hydrolysis of natural fats and oil during the biodiesel production or by the fermentation of sugars [4, 5]. Biosynthesis of 1,3-PD from glycerol has been extensively studied in species of *Klebsiella*, *Clostridia*, *Citrobacter*, *Enterobacter*, and *Lactobacillus* [6]. However, some disadvantages still hinder the industrialization of 1,3-PD fermentation, such as low concentration, yield, and productivity of 1,3-PD, relatively high byproduct production, and high residual glycerol concentration in the broth [7]. The aim of the present study was to investigate the effect of pH control strategy on 1,3-PD production by fed-batch culture of *Klebsiella pneumoniae* to establish an economical 1,3-PD fermentative production process.

Materials and Methods

Strain and Medium

K. pneumoniae ME-308 that was insensitive to O_2 screened from *K. pneumoniae* ATCC 25955 was used.

The seed medium contained (g/l): yeast extract, 1.5; malt, 1.5; peptone, 2.5; NaCl, 2.5 [8].

The fermentation medium contained (g/l): yeast extract, 5.0; $K_2HPO_4 \cdot 3H_2O$, 10; KH_2PO_4, 2.0; NH_4Cl, 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.03; $CoCl_2 \cdot 6H_2O$, 0.005; vitamin B_{12}, 0.005; and glycerol, 50.0 [8].

Cultivation Conditions

The seed cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml medium at 37 °C for 12 h with shaking at 200 rpm and subsequently inoculated into the bioreactor at 5% (*v*/*v*). The fermentable cultivation was carried out in a 3-l stirred-vessel bioreactor (BioFlo 110, New Brunswick Scientific, USA) containing 2-l fermentation medium. All the fermentation experiments were carried out at 37 °C and 200 rpm. A micro-aerobic environment in the bioreactor was maintained by aerating air at 0.1 vvm during the first 5 h, and then no air was sparged. In fed-batch fermentation, the limiting substrate, glycerol, was initially of 50 g/l at the beginning of fermentation, and was then fed to the bioreactor during the course of cultivation. Thirty percent (*v*/*v*) ammonia and 30% (*v*/*v*) sulphuric acid were used for pH control.

Analytical Methods

Biomass was determined by measuring turbidity at 590 nm with appropriate dilution using a UV-visible spectroscopy system (DU-640, Beckman, USA). The value of the optical density was converted to dry cell weight (DCW) using a calibration equation ($DCW = 1.2058 \times OD_{590} - 0.0608$).

Glycerol, 1,3-PD, 2,3-butanediol (2,3-BD), ethanol, lactic acid, and acetic acid 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, 0.8 ml•min^{-1}; and column temperature, 65 °C.

Results and Discussion

pH-Stat Fed-batch Culture with Separate Glycerol and Ammonia

The previous work indicated that in the batch fermentation, with initial high substrate concentration, the conversion became slow and glycerol remained in the culture at the end of the fermentation, which would make downstream separation process more difficult as the boiling points and chemical properties of 1,3-PD and glycerol were close to each other [9–11]. Again, the accompanying formed acids such as lactic and acetic acid synthesized by the oxidative pathway in *K. pneumoniae* could cause the culture pH decreasing remarkably during the fermentation and thus inhibited cell growth. Therefore, it was important to keep glycerol concentration and the broth pH value within a proper range to eliminate the inhibition of substrate and byproducts. A kind of pH-stat fed-batch culture was therefore proposed in which glycerol and ammonia were fed in separate lines to keep the glycerol in a comparatively lower concentration and the medium pH equal to the set-point during the fermentation. In Fig. 1a, glycerol was added at 18 and 24 h respectively, and the final concentration of 1,3-PD was 26.65 g/l at 32 h with a yield of 0.62 mol/mol and the productivity of 0.83 g/l/h. At 28 h, the growth of *K. pneumoniae* became slow as shown by the DCW and thus the production of 1,3-PD was ceased. This was due to that the addition rate of glycerol was faster than its consumption rate, which allowed the glycerol concentration to go above the critical concentration of 50.0 g/l. Thus, the cells seemed to turn into a stationary phase and hibernate quickly after 28 h and thus no 1,3-PD was generated then. Furthermore, the concentrations of lactic acid, 2,3-BD and ethanol started to increase greatly after 15 h, with a final concentration as high as 5.48 g/l, 2.4 g/l, and 4.15 g/l, respectively. While as shown in Fig. 1b, the addition of glycerol could not keep up with its consumption. At 27 h, additional glycerol was added when glycerol in the vessel was nearly exhausted. The production of 1,3-PD could not be resumed for the reason that the cells were kept in low glycerol concentration for too long and could not be adaptive to the relatively high glycerol concentration.

Since the feeding occasion was not so easy to determine due to the lack of glycerol online analysis equipment, the above pH-stat fed-batch culture with separate glycerol and ammonia proved to be not so efficient for highly accumulation of 1,3-PD. So designing a proper feeding strategy which could keep the cell growth without inhibition and limitation by glycerol and meanwhile keep the pH value stay to be at an optimal set-point would be a straightforward way for efficient 1,3-PD production.

pH-Stat Fed-batch Culture with Glycerol–Ammonia Mixture

In order to avoid determining proper feeding occasion for the pH-stat fed-batch culture with separate glycerol and ammonia, a modified pH-stat fed-batch culture was designed. In this course, the culture pH was controlled by feeding the glycerol–ammonia mixture (3 vol of glycerol mixing with 1 vol of 30% (*v*/*v*) ammonia), at the same time the glycerol was supplemented into the culture coupled with the pH set-point and was kept between 30–50 g/l which was suitable for both cell growth and 1,3-PD production. In our previous work [9], the optimal pH for cell

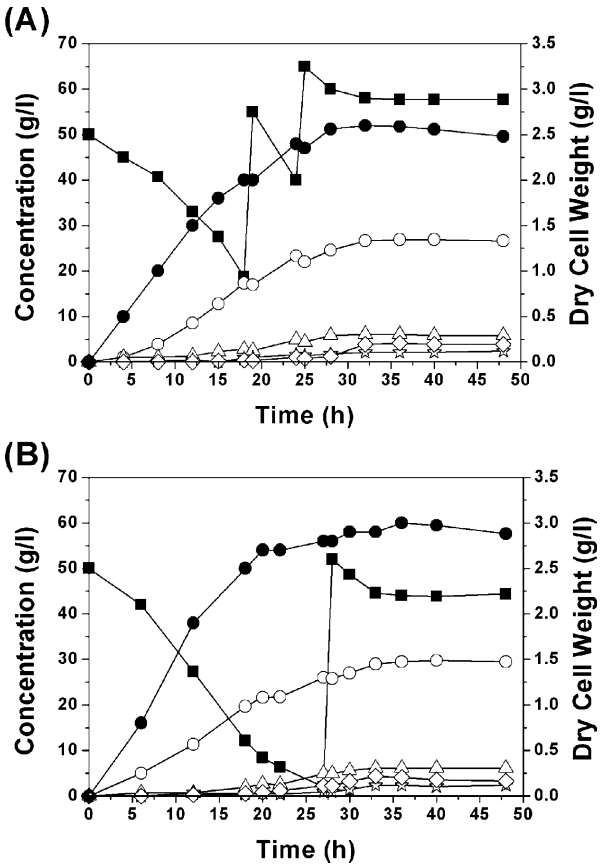

Fig. 1 Time course of 1,3-propanediol production by pH-stat fed-batch culture of *K. pneumoniae* with separate glycerol and ammonia. **a** Glycerol was fed at a comparatively early occasion (pH 7.1), **b** glycerol was fed at a comparatively late occasion (pH 7.1). The *symbols* were used: glycerol (*filled square*), 1,3-propanediol (*unfilled circle*), lactic acid (*unfilled triangle*), ethanol (*unfilled diamond*), 2,3-butanediol (*unfilled star*), and dry cell weight (*filled circle*)

growth of *K. pneumoniae* ME-308 proved to range from 6.3 to 7.3, thus two different pH values in this modified pH-stat fed-batch culture were tested at 6.3 and 7.3, respectively.

As shown in Fig. 2a, when the pH value was set to be 7.3, a great deal of lactic acid appeared after 40 h, reaching 11.2 g/l, comparing with the final 1,3-PD concentration of 51.8 g/l. When the culture pH was constant to be 6.3, it could be noticed that as the main byproduct, excessive 2,3-BD had been generated instead of lactic acid. The final 2,3-BD concentration reached 17.5 g/l, comparing with a final 1,3-PD concentration of 47.5 g/l (Fig. 2b). In these two cases, the intent to get a higher 1,3-PD final concentration was not successfully fulfilled for that lactic acid and 2,3-BD which both competed NADH with 1,3-PD formation [3] were comparatively highly produced at the same time respectively. Also, there was about 20 g/l of residual glycerol at the end of fermentation, which would cause more difficulty in latter separation of 1,3-PD from the broth [12].

The above results demonstrated that the modified pH-stat fed-batch culture was also not able to produce 1,3-PD efficiently. Although the substrate inhibition could be eliminated by controlling the glycerol concentration in a relatively fixed range through coupled successive glycerol feeding and pH controlling, at the end of the fermentation, the relatively high concentration of competing byproduct (2,3-BD or lactic acid) and residual glycerol would lower the yield of 1,3-PD from glycerol and cause difficulty for the downstream processing respectively, and thus limit the development of an economical 1,3-PD fermentation. So this feeding strategy still need to be improved.

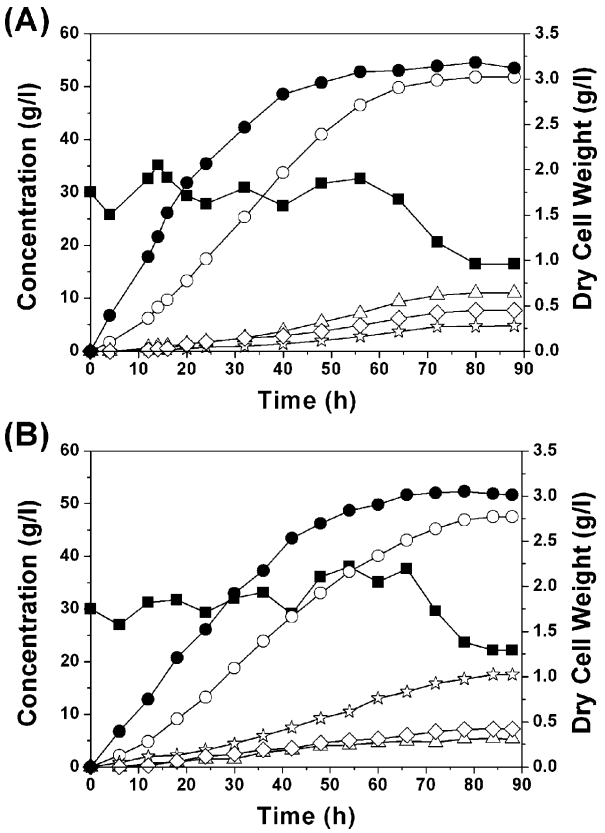

Fig. 2 Time course of 1,3-propanediol production by pH-stat fed-batch culture of *K. pneumoniae* with glycerol–ammonia mixture at **a** pH 7.3, **b** pH 6.3. The *symbols* were used: glycerol (*filled square*), 1,3-propanediol (*unfilled circle*), lactic acid (*unfilled triangle*), ethanol (*unfilled diamond*), 2,3-butanediol (*unfilled star*), and dry cell weight (*filled circle*)

pH-Fluctuating Fed-batch Culture with Glycerol–Ammonia Mixture and Sulphuric Acid

In Fig. 2, an important phenomenon was observed that the yield of two main byproducts, 2,3-BD and lactic acid were closely related to pH value. pH 6.3 favored 2,3-BD formation while pH 7.3 favored lactic acid formation. Generally, as shown in Fig. 3, when pH was at 5.0 to 6.5, the main byproduct was 2,3-BD and the concentration of lactic acid was low. However, when pH was 7.1 to 8.0, the major byproduct changed to be lactic acid. And the reason why 2,3-BD became the main byproduct instead of lactic acid at weak acidic condition (pH 6.3) might be that 2,3-BD was less toxic to microbial systems than lactic acid, and the key enzyme involved in 2,3-BD formation pathway, namely α-acetolactate synthase, was induced under weak acidic condition [13], also as indicated by Zeng et al. [14], 2,3-BD production was optimum between pH 5.5 and 6.5 and dropped off at a higher pH. While when the broth became weak alkaline (pH 7.3), the dominant byproduct turned to be lactic acid and this could be explained by the fact that the undissociated organic acids were usually more toxic than their dissociated forms [15–17]. The critical concentration of lactic acid totally inhibiting the growth of *K. pneumoniae* had been reported to be about 10 g/l [18], compared with the data reported in our precious study in a total inhibition by a lactate concentration of 70 g/l at pH 7.1 [9]. When pH was higher than 7.0, most lactic acid in the broth changed to be in the form of lactate, which was less toxic to the cell than lactic acid. At the same time, as 2,3-BD formation pathway was inactive at this weak alkaline

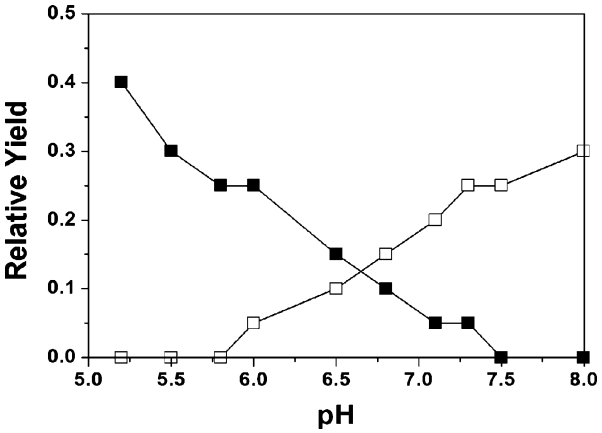

Fig. 3 Effect of pH on the production of 2,3-butanediol and lactic acid. The relative yield of 2,3-butanediol (*filled square*) and lactic acid (*unfilled square*) was defined as $C_{2,3\text{-BD}}/C_{1,3\text{-PD}}$ and $C_{\text{Lactic acid}}/C_{1,3\text{-PD}}$ respectively. The final yields of 2,3-butanediol and lactic acid according to the pH change are shown based on the data from batch fermentations with the initial glycerol concentration of 50 g/l

condition, lactic acid in the form of less toxic lactate became the dominant byproduct. In other words, under this condition, the metabolic mechanism preferred to use the lactic acid pathway to regenerate NAD^+, instead of the 2,3-BD pathway. So it was concluded that the switching of metabolic pathways to respond to environmental pH changes in *K. pneumonia* must be caused by a kind of intrinsic self-protection mechanism of bacteria.

To avoid either of the two competing byproduct excessive formation, a new kind of pH control strategy was developed based on the bacterial self-protection mechanism during which glycerol–ammonia mixture (3 vol of glycerol mixing with 1 vol of 30% (*v*/*v*) ammonia) and 30% (*v*/*v*) sulphuric acid were used to fluctuate pH value between 6.3 and 7.3 periodically. In Fig. 4a, the initial pH was controlled at 7.3, but shifted to 6.3 after 16 h, then switched back to 7.3 after 16 h and cyclically fluctuated between 7.3 and 6.3 every 32 h until the end of the fermentation. It was noticed that 1,3-PD yield from glycerol was as high as 0.73 mol/mol, with a high productivity of 0.81 g/l/h and a high final 1,3-PD concentration of 64.5 g/l in this situation, and either of 2,3-BD or lactic acid was kept under 5 g/l (Table 1). Further experiment with the starting pH value of 6.3 and the cycle for the pH fluctuation changed to be 16 h was conducted (Fig. 4b). And at the end of fermentation, the yield of 1,3-PD from glycerol was 0.70 mol/mol, with the productivity of 0.97 g/l/h and the final 1,3-PD concentration was as high as 70 g/l with both 2,3-BD and lactic acid kept under 5 g/l (Table 1). In addition, by applying this pH-fluctuating fed-batch strategy, the residual glycerol in the broth at the end of fermentation was encouragingly very low (Table 1), and this would decrease the difficulty in the downstream separation process. However, the yield of 1,3-PD in Fig. 4b was lower than the yield in Fig. 4a. On the other hand, the final 1,3-PD concentration in Fig. 4b was higher than the concentration in Fig. 4a. The problem arose in part from different pH-fluctuating parameters such as the length of cycle and initial pH value which were closely related to cell growth and metabolism.

In a word, under this kind of pH-fluctuating fed-batch culture, the metabolic pathways of the two competing byproducts 2,3-BD and lactic acid were sub-active for the periodical low or high pH stress. Lactic acid formation was inhibited under weak acidic condition (pH 6.3), while 2,3-BD formation was inhibited when the culture condition turned to be weak alkaline (pH 7.3), this made the carbon flux flow into the object product 1,3-PD, and thus the yield and productivity of 1,3-PD were enhanced.

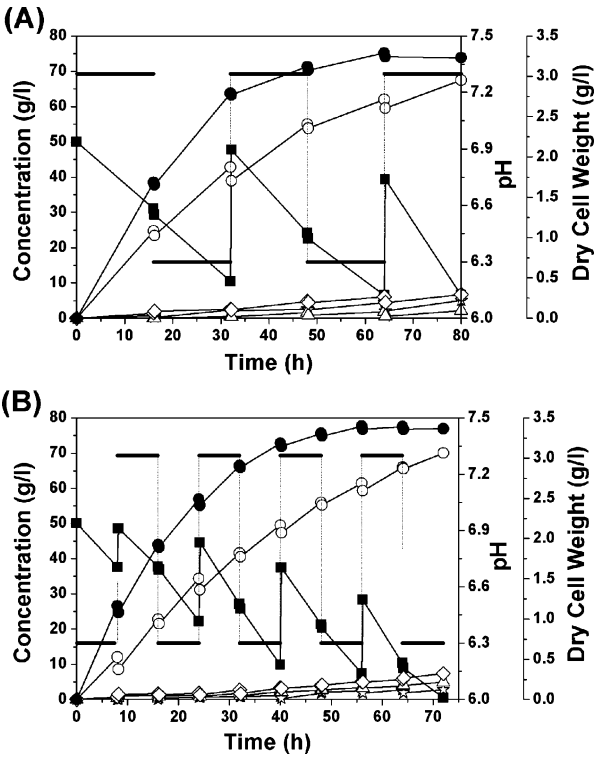

Fig. 4 Time course of 1,3-propanediol production by pH-fluctuating fed-batch culture of *K. pneumoniae* with glycerol–ammonia mixture and sulphuric acid. **a** initial pH 7.3, fluctuating cycle: 32 h, **b** initial pH 6.3, fluctuating cycle: 16 h. The symbols were used: glycerol (*filled square*), 1,3-PD (*unfilled circle*), lactic acid (*unfilled triangle*), ethanol (*unfilled diamond*), 2,3-butanediol (*unfilled star*), dry cell weight (*filled circle*), and pH (*horizontal line*)

Table 1 The analysis of parameters at different pH control strategies during the fed-batch culture.

Parameters	pH-stat				pH-fluctuating	
	A[a]	B[a]	C[b]	D[c]	A[d]	B[e]
Cycle for pH fluctuation (h)	–	–	–	–	32	16
Fermentation time (h)	32	33	80	84	80	72
1,3-propanediol (g/l)	26.65	29.50	51.80	47.50	64.50	70.00
Lactic acid (g/l)	5.84	6.10	11.20	5.30	2.20	4.90
2,3-butanediol (g/l)	2.40	2.35	4.80	17.50	3.70	2.80
Ethanol (g/l)	4.15	4.36	7.70	7.20	6.70	7.35
Glycerol exhausted (g/l)	51.30	55.65	94.10	87.95	107.50	120.60
Residual glycerol (g/l)	57.70	44.33	16.50	21.13	2.60	0.50
1,3-propanediol yield (mol/mol)	0.62	0.64	0.67	0.65	0.73	0.70
1,3-propanediol productivity (g/l/h)	0.83	0.89	0.65	0.57	0.81	0.97

Glycerol–ammonia mixture: 3 vol of glycerol mixing with 1 vol of 30% (*v*/*v*) ammonia

[a] pH was controlled at 7.1 by feeding with separate 80% (*v*/*v*) glycerol and 30% (*v*/*v*) ammonia at a initial glycerol concentration of 50 g/l

[b] pH was controlled at 7.3 by feeding with glycerol–ammonia mixture with initial glycerol concentration of 30 g/l

[c] pH was controlled at 6.3 by feeding with glycerol–ammonia mixture with initial glycerol concentration of 30 g/l

[d] pH was fluctuated between 6.3 and 7.3 every 32 h by feeding with glycerol–ammonia mixture and 30% (*v*/*v*) sulphuric acid with initial glycerol concentration of 50 g/l and pH value of 7.3

[e] pH was fluctuated between 6.3 and 7.3 every 16 h by feeding with glycerol–ammonia mixture and 30% (*v*/*v*) sulphuric acid with initial glycerol concentration of 50 g/l and pH value of 6.3

Conclusions

In this paper, three different pH control coupling fed-batch strategies were employed to improve the production of 1,3-PD by *K. pneumoniae*. Based on the analysis of the product profiles under different pH values, a self-protection mechanism in *K. pneumoniae*, namely that the growing *K. pneumoniae* cells switched the metabolic pathways responding to environmental pH changes, was proposed. Thus, a new kind of feeding strategy was further developed based on the mechanism during which pH value fluctuated between 6.3 and 7.3 periodically by feeding glycerol–ammonia mixture and sulphuric acid. This kind of feeding strategy not only efficiently limited the byproducts production, but also successfully reduced the residual glycerol concentration in the broth. At last, the maximum concentration of 1,3-PD reached 70 g/l with the byproducts and residual glycerol under low concentrations. And the proposed pH-fluctuating fed-batch culture proved to be an effective method for economical 1,3-PD production.

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