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Microbial Production of 1,3-Propanediol by *Klebsiella pneumoniae* XJPD-Li under Different Aeration Strategies

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Abstract The microbial production of 1,3-propanediol (1,3-PD) by *Klebsiella pneumoniae* XJPD-Li under different aeration strategies were investigated. In batch fermentation, the results showed that the final concentration of 1,3-PD and yield on glycerol were 13.44 g/l and 0.73 mol/mol under the anaerobic condition (N_2, 0.4 vvm), 11.55 g/l and 0.62 mol/mol without aeration, and 8.73 g/l and 0.47 mol/mol under the aerobic condition (air, 0.4 vvm), respectively. Under the aerobic condition, the yield of 1,3-PD on glycerol was the lowest, while the biomass (optical density at 650 nm) was the highest among these three conditions. In the fed-batch culture, the final concentration and the yield of 1,3-PD was 60.82 g/l and 0.61 mol/mol under the anaerobic condition (N_2, 0.4 vvm), 56.43 g/l and 0.53 mol/mol without aeration, and 65.26 g/l and 0.56 mol/mol under the aerobic condition. All these three conditions had good productivities of 1,3-PD, which were 3.35 g/l·h under the anaerobic condition (N_2, 0.4 vvm), 3.13 g/l·h without aeration, and 3.16 g/l·h under the aerobic condition within the initial 12 h.

Keywords Fermentation · Glycerol · 1,3-Propanediol · *Klebsiella pneumoniae* XJPD-Li · Aeration

Introduction

1,3-Propanediol (1,3-PD) has numerous applications in polymers, cosmetics, foods, lubricants, and medicines. The industrial production of 1,3-PD has attracted attention as an important monomer to synthesize a new type of polyester, polytrimethylene terephthalate [1]. However, 1,3-PD has been produced chemically either by the hydratation of acrolein or

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by the hydroformylation of ethylene. The microbial production of 1,3-PD, a socially beneficial route to obtain chemicals from renewable resources, has been widely researched and considered as a competitor to the traditional petrochemical routes [2]. Among the microorganisms that can convert glycerol to 1,3-PD, *Klebsiella pneumoniae*, *Clostridium butyricum*, and *Citrobacter freundii* are paid more attention because of their appreciable substrate tolerance, yield, and productivity [2]. Biological efforts include fermentation optimization of the natural glycerol-utilizing process and an ambitious metabolic engineering to increase the concentration and productivity of 1,3-PD [3].

The glycerol metabolism of the model organism *K. pneumoniae* involves two branch pathways: the oxidative branch and the reductive branch [4]. In the oxidative branch, glycerol is transformed to dihydroxyacetone by glycerol dehydrogenase, which then undergoes normal glycolysis to form pyruvate and finally can be converted into various byproducts such as acids and alcohols. Glycerol is converted in the reductive pathway by a coenzyme B_{12}-dependent glycerol dehydratase (GDHt) to 3-hydroxypropionaldehyde, which is then reduced by NADH to 1,3-PD with 1,3-PD–NAD oxidoreductase as the catalyst [5, 6]. The second metabolic pathway, which maintains the redox balance of the cell, is essential to convert glycerol to 1,3-PD, and GDHt is the key limited enzyme for this biological process.

Commonly, the biosynthesis of 1,3-PD is always done under anaerobic conditions. According to recent reports, 1,3-PD can also be obtained under microaerobic or low aeration conditions. The concentration, molar yield, and productivity of 1,3-PD in fed-batch fermentation under microaerobic conditions were 59.50 g/l, 51.57%, and 1.57 g/l·h, respectively [7–9]. Comparing with other strains, *K. pneumoniae* XJPD-Li isolated from Xinjiang province, China, could biosynthesize 1,3-PD with a higher molar yield and shorter fermentation time at a higher cultivation temperature, which was stored in our laboratory. The molar yield of 70% was achieved in fed-batch during 48 h of fermentation [10]. The aim of this study was to investigate the effect of aeration conditions on 1,3-PD production by *K. pneumoniae* XJPD-Li; thus, different aeration strategies had been carried out in batch and fed-batch cultures, and the metabolic fluxes of glycerol in *K. pneumoniae* XJPD-Li were analyzed.

Materials and Methods

Strain and Medium

K. pneumoniae XJPD-Li was obtained from the Key Laboratory for Green Processing of Chemical Engineering of Xinjiang Bingtan, Shihezi University, People's Republic of China.

The seed medium contained (g/l): $K_2HPO_4{\cdot}3H_2O$, 4.4; KH_2PO_4, 1.3; $(NH4)_2SO_4$, 2.0; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCO_3$, 2; yeast extract, 1.0; glycerol, 20.0; 1 ml/l trace element solution; and 2 ml/l Fe solution [8].

The fermentation medium contained (g/l): $K_2HPO_4{\cdot}3H_2O$, 5.1; KH_2PO_4, 1.5; $(NH4)_2SO_4$, 6.0; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.02; yeast extract, 1.0; glycerol, 20.0; 1 ml/l trace element solution; and 2 ml/l Fe solution.

Cultivation Conditions

The seed cells were grown in a 250-ml shake flask containing 100 ml medium at 40 °C for 12 h and subsequently inoculated into the bioreactor at 10% (*v*/*v*). The fermentable

cultivation was carried out in a 5-l stirred-vessel bioreactor (Bioflo 110, New Brunswick Scientific, USA) containing 3 l fermentation medium. The pH was controlled by addition of 5 M potassium hydroxide automatically together with glycerol (50%), and all fermentation experiments were carried out at 40 °C and 200 rpm. An anaerobic or aerobic environment in the bioreactor was maintained by aerating nitrogen or air at 0.4 vvm.

Analytical Procedures

The biomass was estimated by measuring optical density at 650 nm (OD_{650}).

The protein was assayed by the method of Bradford [11] with crystalline bovine serum albumin as a standard. Specific activity is expressed as units per milligram of protein.

Glycerol, 1,3-PD, acetate, succinate, lactate, and ethanol were analyzed by a high-performance liquid chromatography system (SHIMADZU 10A) equipped with a differential refractive index detector and an Aminex HPX-87H column (Bio-Rad) at 65 °C with 0.005 M H_2SO_4 as a mobile phase at 0.8 ml/min.

GDHt activity was determined by the modified 3-methyl-2-benzothiazolinone hydrazone (MBTH) method according to Toraya et al. [12].The reaction mixture (3.5 ml) was composed of 0.05 M KCl (0.1 ml), 0.2 M 1,2-propanediol(0.1 ml), 15 μM adenosylcobalamin (0.1 ml), and 0.7 ml cell extraction. After incubation at 40 °C for 10 min, the enzyme reaction was stopped by adding 1.0 ml of 0.1 M potassium citrate buffer and 0.5 ml of 0.1% MBTH solution. After 15 min at 40 °C, 1.0 ml of water was added, and the absorbance was measured at 305 nm. One unit of GDHt is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde per minute at 40 °C [10].

Results and Discussion

Effect of Aeration Conditions on 1,3-PD Production in Batch Culture

Table 1 shows the effect of aeration conditions in batch cultures by *K. pneumoniae* XJPD-Li. Glycerol was used up at 8 h in the fermentation course (Not shown) under three strategies. Under the aerobic condition, 1,3-PD was also detected in the culture medium, which indicated that the metabolic pathway of *K. pneumoniae* XJPD-Li from glycerol to 1,3-PD was not entirely inhibited. The highest concentration, yield, and productivity of 1,3-PD were obtained under the anaerobic condition, while those under the aerobic condition were the lowest. The results (concentration, yield, and productivity of 1,3-PD) without aeration were between the anaerobic and aerobic conditions. Moreover, the biomass was the highest under the aerobic condition. Aerating air is propitious to the expression of the enzymes in the citric acid cycle, which provides much energy for the growth of cells [7, 9].

Table 1 Results of batch cultures under different aeration conditions at 12 h.

Condition	Biomass (OD_{650})	1,3-PD concentration (g/l)	1,3-PD yield (mol/mol)	1,3-PD productivity (g/l·h)
Anaerobic	1.64	13.44	0.73	1.12
Without aeration	1.56	11.55	0.62	0.96
Aerobic	2.23	8.73	0.47	0.73

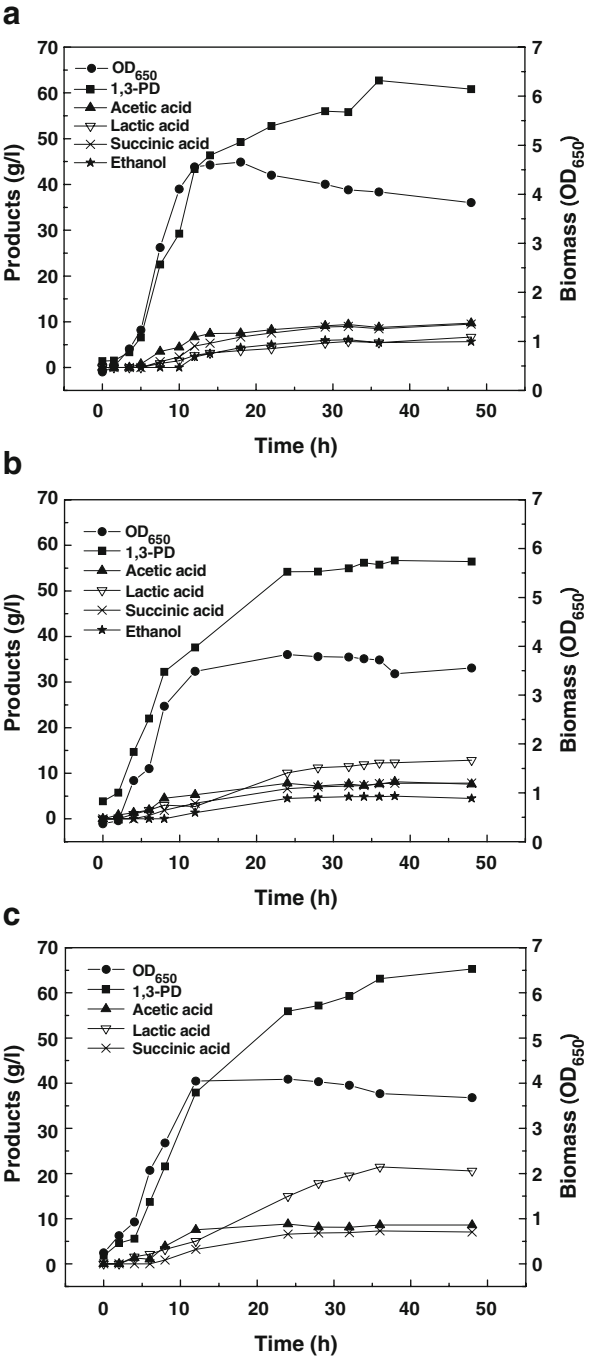

Fig. 1 Time course of biomass and concentration of products in the fed-batch fermentation by *K. pneumoniae* XJPD-Li under different aeration conditions: **a** N_2 0.4 vvm, **b** without aeration, **c** air 0.4 vvm

This explains the fast growth of cells and decreasing yield of 1,3-PD from glycerol in batch culture of *K. pneumoniae* XJPD-Li.

Effect of Aeration Conditions on 1,3-PD Production in Fed-batch Culture

The results of fed-batch fermentation by *K. pneumoniae* XJPD-Li under different aeration conditions were shown in Fig. 1. The anaerobic fed-batch fermentation had a good productivity, approximately 3.3 g/l·h in 12 h with a final concentration of 1,3-PD 60.82 g/l, and the yield, 0.61 mol/mol glycerol. There were also some fermentation byproducts, which included acetic acid (9.72 g/l), lactic acid (6.67 g/l), succinic acid (9.48 g/l), and ethanol (5.65 g/l; Fig. 1a).

The fermentation without aeration had a final concentration of 1,3-PD 56.43 g/l and a yield of 1,3-PD 0.53 mol/mol to glycerol. Lactic acid concentration was 12.85 g/l, which doubled that under the anaerobic condition. The concentrations of acetate, succinate, and ethanol were 7.61, 7.85, and 4.49 g/l, respectively (Fig. 1b). It was worth pointing out that the dissolved oxygen concentration decreased rapidly to a low level (about 2–5%) in the first 12 h.

In the aerobic, fed-batch fermentation, 1,3-PD concentration increased linearly in the initial 36 h. A high concentration of 1,3-PD (65.26 g/l) was obtained within 48 h (Fig. 1c), leading to a yield of 0.56 mol/mol. In this case, the concentration of lactic acid was 20.57 g/l, which was twice more than that under the anaerobic condition. In addition, ethanol was not detected.

As shown in Table 2, the biosynthesis of 1,3-PD mainly occurred in the first two stages (0–24 h) and the productivity of 1,3-PD under different aeration strategies decreased gradually. The productivities under the anaerobic and aerobic conditions were 7.6% and 15.3%, which were higher than that without aeration (0–48 h). This indicated that aerating a certain volume of air or N_2 was beneficial to 1,3-PD production. The best one might be from combining the two strategies together, which was to keep the fermentation condition anaerobic in the first stage (0–12 h) and then aerobic in the next stage (12–48 h). An anaerobic/aerobic-combined fed-batch culture was developed giving 70 g/l 1,3-PD and 16 g/l 2,3-butanediol with a total diol yield of 0.6 mol/mol glycerol [8].

Effect of Aeration Conditions on GDHt Activity

The effect of aeration conditions on GDHt activity in batch culture (8 h) and fed-batch culture (36 h) were shown in Fig. 2. Under any condition, there was always GDHt activity detected in the culture of *K. pneumoniae* XJPD-Li. It was due to the low level of dissolved oxygen concentration. In the batch culture, the GDHt activity without aeration was almost the same as that under the anaerobic condition but not under the aerobic condition, which was 32% lower than that under the anaerobic condition. However, there was no distinct difference in the fed-batch culture. The activities were all around 0.7 U/mg.

Table 2 Productivities at different stages of 1,3-PD fed-batch fermentations by *K. pneumoniae* XJPD-Li under different aeration strategies.

Fermentation time (h)	1,3-PD productivity (g/l·h)		
	Anaerobic	Without aeration	Aerobic
0–12	3.35	3.13	3.16
12–24	1.03	1.38	1.50
24–48	0.34	0.10	0.39
0–48	1.27	1.18	1.36

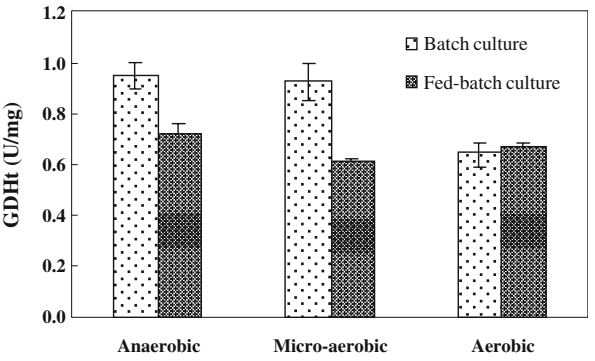

Fig. 2 Effect of aeration conditions on GDHt activity

Effect of Aeration Conditions on Metabolic Fluxes of Glycerol

The formation of 1,3-PD in glycerol fermentation was considered as the regeneration of NAD^+ in which the necessary reducing equivalents, as well as the necessary energy, have to be produced by oxidation of glycerol to other products [13, 14]. Figure 3 showed the substrate channeling and product formation under different aeration strategies in the fed-batch culture of *K. pneumoniae* XJPD-Li. With the condition changed from anaerobic to aerobic, the flux from glycerol to the reductive branch reduced, while the flux to the oxidative branch increased. The fluxes to biomass were all around 2%, which can be used for 1,3-PD production in that reducing equivalents can also be produced in biomass growth.

Major changes were observed in the fluxes around pyruvate. According to [1], only acetic acid as the byproduct gives the highest 1,3-PD yield namely, 64% of the glycerol consumed. In the course of cell growth, the acetate concentration was in the range of

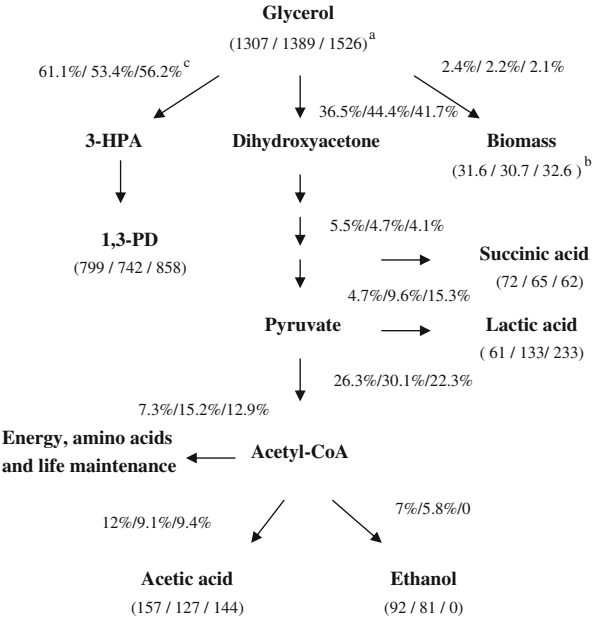

Fig. 3 Substrate channeling and product formation under different aeration strategies in the fed-batch culture of *K. pneumoniae* XJPD-Li. **a** Final molar titer of glycerol consumed under anaerobic/without aeration/ aerobic conditions (mmol/l); **b** yield of biomass, the molar concentration is calculated based on previous article [13]; **c** molar fractions of glycerol fluxes in overall glycerol consumed

5.0–6.2 g/l in the fed-batch culture of *K. pneumoniae* [15]. In our work, the corresponding values were 6.66, 5.28, and 7.57 g/l, respectively, at the 12th hour, which was just at the beginning of the stationary phase (Fig. 1a–c). At the same time, a higher concentration of 1,3-PD was obtained under the aerobic condition than that of both the anaerobic condition and without aeration in the end.

Ethanol is a key competitor in the production of 1,3-PD by *K. pneumoniae* [16, 17]. By the inactivation of aldehyde dehydrogenase in *K. pneumoniae* YMU2, the final titer, the productivity of 1,3-PD, and yield of 1,3-PD were much higher than those in the parent strain [18]. In our research, the flux to ethanol was 0 by aerating 0.4 vvm air in the fed-batch culture, while 1,3-PD yield was not changed obviously for that more glycerol fluxes flowed to energy, amino acids, and life maintenance.

The effect of lactic acid on cell growth and biosynthesis of 1,3-PD by *K. pneumoniae* would not be affected even in 10 g/l lactic acid [19]. In the fermentation of *K. pneumoniae* XJPD-Li, the final concentration of lactic acid reached 20.57 g/l under the aerobic condition (Fig. 1c). However, more glycerol was consumed during lactic acid production under the aerobic condition than that under the anaerobic condition. To improve the concentration and yield of 1,3-PD by *K. pneumoniae* XJPD-Li, further measures of decreasing production of lactic acid should be taken by gene engineering and metabolic engineering.

Conclusions

The batch and fed-batch fermentations of glycerol by *K. pneumoniae* XJPD-Li showed that the aerobic condition was feasible to produce 1,3-PD. Similar to the anaerobic fermentation, the aerobic culture had a final concentration of 1,3-PD 65.26 g/l in the fed-batch fermentation. This is beneficial to the industrial production of 1,3-PD by saving the cost for conduction of the anaerobic condition. In the presence of O_2, however, the metabolic pathway from glycerol to ethanol was inhibited, and more lactic acid was accumulated. The research also demonstrated that *K. pneumoniae* XJPD-Li was an excellent 1,3-PD producer especially in the first 12 h (approx.3.16 g/l·h), and the engineering approaches were essential to improving the yield of 1,3-PD further.

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References

1. Biebl, H., Menzel, K., Zeng, A. P., & Deckwer, W. D. (1999). Microbial production of 1, 3-propanediol. *Applied Microbiology and Biotechnology*, *52*, 289–297.
2. Zhao, Y. N., Chen, G., & Yao, S. J. (2006). Microbial production of 1,3-propanediol from glycerol by encapsulated *Klebsiella pneumoniae*. *Biochemical Engineering Journal*, *32*, 93–99.
3. Nakamura, C. E., & Whited, G. M. (2003). Metabolic engineering for the microbial production of 1,3-propanediol. *Current Opinion in Biotechnology*, *14*, 454–459.
4. Zheng, P., Wereath, K., Sun, J. B., Heuvel, J., & Zeng, A. P. (2006). Overexpression of genes of the *dha* regulon and its effects on cell growth, glycerol fermentation to 1,3-propanediol and plasmid stability in *Klebsiella pneumoniae*. *Process Biochemistry*, *41*, 2160–2169.
5. Tobimatsu, T., Azuma, M., Matsubara, H., Takatori, H., Niida, T., Nishimoto, K., et al. (1996). Cloning, sequence, and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of *Klebsiella pneumoniae*. *Journal of Biological Chemistry*, *271*, 22352–22357.

6. Rolf, D., Bobik, T. A., & Gottschalk, G. (1999). Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organization of the encoding genes. *FEMS Microbiology Reviews*, *22*, 553–566.
7. Chen, X., Zhang, D. J., Qi, W. T., Gao, S. J., Xiu, Z. L., & Xu, P. (2003). Microbial fed-batch production of 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions. *Applied Microbiology and Biotechnology*, *63*, 143–146.
8. Cheng, K. K., Liu, D. H., Sun, Y., & Liu, W. B. (2004). 1, 3-Propanediol production by *Klebsiella pneumoniae* under different aeration strategies. *Biotechnology Letters*, *26*, 911–915.
9. Lin, R. H., Liu, H. J., Sun, Y., Cheng, K. K., & Liu, D. H. (2006). Effect of oxygen on the metabolism of *Klebsiella pneumoniae* in 1,3-propanediol fermentation(Chinese). *The Chinese Journal of Process Engineering*, *6*(1), 96–99.
10. Zhang, G. L., Ma, B. B., Xu, X. L., Li, C., & Wang, L. W. (2007). Fast conversion of glycerol to 1,3-propanediol by a new strain of *Klebsiella pneumoniae*. *Biochemical Engineering Journal*, *37*, 256–260.
11. Bradford, M. M. (1976). A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein dye building. *Analytical Biochemistry*, *72*, 248–254.
12. Toraya, T., Kuno, S., & Fukui, S. (1980). Distribution of coenzyme B12-dependent diol dehydratase and glycerol dehydratase in selected genera of *Enterobacteriaceae* and *Propionibacteriaceae*. *Journal of Bacteriology*, *141*, 1439–1442.
13. Zeng, A. P., Biebl, H., & Schlieker, H. (1993). Pathway analysis of glycerol fermentation by *K. pneumoniae*: regulation of reducing equivalent balance and product formation. *Enzyme and Microbial Technology*, *15*, 770–779.
14. Menzel, K., Ahrens, K., Zeng, A. P., & Deckwer, W. D. (1998). Kinetic, dynamic, and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture: IV. Enzymes and fluxes of pyruvate metabolism. *Biotechnology and Bioengineering*, *60*, 617–622.
15. Hartlep, M., Hussmann, W., Prayitno, N., Meynial-Salles, I., & Zeng, A. P. (2002). Study of two-stage processes for the microbial production of 1,3-propanediol from glucose. *Applied Microbiology and Biotechnology*, *60*, 60–66.
16. Menzel, K., Zeng, A. P., & Deckwer, W. D. (1997). High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae*. *Enzyme and Microbial Technology*, *20*, 82–86.
17. Zhang, Y. P., Du, C. Y., Rao, Z., & Cao, Z. A. (2005). Regulation of vitamin C and vitamin E on the biosynthesis of 1,3-propanediol by *Klebsiella pneumoniae*. *The Chinese Journal of Process Engineering*, *5*, 197–200.
18. Zhang, Y. P., Li, Y., Du, C. Y., Liu, M., & Cao, Z. A. (2006a). Inactivation of aldehyde dehydrogenase: A key factor for engineering 1,3-propanediol production by *Klebsiella pneumoniae*. *Metabolic Engineering*, *8*, 578–586.
19. Zhang, Y. P., Liu, M., Du, C. Y., Shen, J. Y., & Cao, Z. A. (2006b). Effect of by-products on cell growth and biosynthesis of 1,3-propanediol by *Klebsiella pneumoniae*. *The Chinese Journal of Process Engineering*, *6*(5), 804–808 (in Chinese).