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Enhanced Reducing Equivalent Generation for 1,3-Propanediol Production Through Cofermentation of Glycerol and Xylose by *Klebsiella pneumoniae*

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Abstract 1,3-Propanediol (1,3-PD) biosynthesis plays a key role in NADH consumption to regulate the intracellular reducing equivalent balance of *Klebsiella pneumoniae*. This study aimed to increase reducing equivalent for enhancing 1,3-PD production through cofermentation of glycerol and xylose. Adding xylose as cosubstrate resulted in more reducing equivalent generation and higher cell growth. In batch fermentation under microaerobic condition, the 1,3-PD concentration, conversion from glycerol, and biomass (OD_{600}) relative to cofermentation were increased significantly by 9.1%, 20%, and 15.8%, respectively. The reducing equivalent (NADH) was increased by 1–3 mg/g (cell dry weight) compared with that from glycerol alone. Furthermore, 2,3-butannediol was also doubly produced as major byproduct. In fed-batch fermentation with xylose as cosubstrate, the final 1,3-PD concentration, conversion from glycerol, and productivity were improved evidently from 60.78 to 67.21 g/l, 0.52 to 0.63 mol/mol, and 1.64 to1.82 g/l/h, respectively.

Keywords 1,3-Propanediol · Cofermentation · *Klebsiella pneumoniae* · Reducing equivalent · Xylose

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 [1]. Biosynthesis of 1,3-propanediol (1,3-PD) with low-cost renewable glycerol as a substrate has attracted worldwide interests because 1,3-PD is used as a versatile

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degradable intermediate compound for the synthesis of heterocycles and a monomer for the production of polymers [2,3].

Currently, the 1,3-PD producers have only been known from bacteria, such as *Klebsiella pneumoniae* [4], *Citrobacter* [5], *Clostridium* [6], and *Lactobacillus* [7]. The glycerol metabolism is well studied with the model organism *K. pneumoniae*. The 1,3-PD pathway from glycerol is associated with the consumption of reducing power NADH to preserve the intracellular reducing equivalent balance of *K. pneumoniae* [8]. Recently, most metabolic engineering have achieved pronounced progresses in manipulating the availability and higher intracellular reducing power [9–11]. However, alteration of intracellular redox balance by genetic manipulation inevitably reduced the glycerol uptake efficiency, inhibited the growth of producer, or gained a limited effect in conversion. It was also demonstrated that the metabolite distribution can be influenced by a change in the NADH/NAD^+ ratio as mediated by the oxidation state of the carbon source used [12]. Hemicellulosic sugars, especially D-xylose, are relatively abundant in agricultural and forestry residues. Moreover, they can be recovered from the hemicelluloses by acid hydrolysis more readily and in better yields than can D-glucose from cellulose. These factors favor hemicellulosic sugars as a feedstock for production of ethanol and other chemicals. Unfortunately, D-xylose is not so readily utilized as D-glucose for the production of chemicals by microorganisms. The reason may lie in the biochemical pathways used for pentose and hexose metabolism. Whereas D-glucose is metabolized by the Embden–Meyerhoff–Parnas pathway, D-xylose metabolism proceeds by way of the pentose phosphate pathway. In general, the pentose metabolism could accumulate a large amount of reducing power (NADPH and NADH) [13]. If such a process of 1,3-PD production from the cofermentation of glycerol and D-xylose is feasible in large application, more economic cofermentation of glycerol and hemicellulosic hydrolysates will be carried out in the future.

In this study, we aimed at increasing internal redox state (the intracellular NADH/NAD^+ level) through cofermentation of glycerol and xylose for improving the 1,3-PD production. As more reducing equivalent is generated, more metabolic flux overflowed to 1,3-PD biosynthesis for the intracellular reducing equivalent balance. By this approach, we also got higher content of cell mass and growth rate which is advantageous to 1,3-PD synthesis.

Materials and Methods

Microorganism

The strain for 1, 3-PD production used in this study was *K. pneumoniae* ME-303, obtained from Nanjing University of Technology. The culture was maintained on Luria–Bertani agar slant at 4 °C.

Media and Cultures

The seed medium contained yeast extract 3.0 g/l, malt 3.0 g/l, peptone5.0 g/l, and NaCl 5.0 g/l. The basic fermentation media was composed of yeast extract 5 g/l, $K_2HPO_4{\cdot}3H_2O$ 10 g/l, KH_2PO_4 2 g/l, NH_4Cl 1 g/l, NaCl 0.5 g/l, $MgSO_4{\cdot}7H_2O$ 0.1 g/l, $FeCl_3{\cdot}6H_2O$ 30 mg/l, $CoCl_2{\cdot}6H_2O$ 5 mg/l, vitamin B_{12} 5 mg/l, and glycerol 30 g/l. The cofermentation medium is based on the fermentation medium and supplemented with 8 g/l xylose.

Erlenmeyer flasks (250 ml) containing 100 ml seed medium were inoculated with *K. pneumoniae* to prepare the inoculums. The flasks were incubated at 37 °C and 140 rpm for 10 h, and subsequently inoculated into the bioreactor at 5% (v/v). The batch cultivations containing the basic fermentation medium or cofermentation medium were both conducted in a 3-l stirred fermenter (BioFlo 100; New Brunswick Scientific Co., NJ, USA) with a working volume of 2 l under 0.25 vvm air flow. All cultivation was carried out at initial pH 7.0, 37 °C, and 300 rpm. The initial xylose was contained 8 g/l in the cofermentation. The fed-batch fermentation was conducted in a 7-l stirred fermenter with a working volume of 4 l under 0.25 vvm air flow. The glycerol and xylose were initially of 30 g/l and 8 g/l, respectively, and contained 15–20 g/l and 5–8 g/l during time courses of cultivation. During the fed-batch fermentation, the pH was controlled at 7.0 by automatic addition of 10 mol/l NaOH.

Substrates and Fermentation Products Analysis

Glycerol, 1,3-PD, acetic acid, 2,3-butanediol (2,3-BD), lactic acid, alcohol, succinic acid, and xylose were assayed using a high-performance liquid chromatography (HPLC) system (Dionex with a Bio-Rad HPX-87H ion exclusion column) with a refractive index detector at 65 °C with 0.005 mol/l H_2SO_4 as mobile phase at 0.8 ml/min.

Biomass was determined by measuring the value of optical density at 600 nm with appropriate dilution using a UV–visible spectroscopy system. The value of the optical density was converted to cell dry weight (CDW) using a calibration equation.

Determination of Nucleotide Pools

The concentration of intracellular NADH and NAD^+ was measured by HPLC (column stable-C18) [14–16]. The buffer consisting of 4.5 ml methanol (60%) and 0.5 ml HEPES (10%) was stored for more than 4 h at −80 °C. A 1.0-ml sample was taken and then frozen in the prepared buffer for 5 min ensuring rapid cooling and effective inhibition of cell metabolism. The mixture was centrifuged for 5 min at 10,000×*g* and 4 °C to collect the precipitate. For extracting the NADH and NAD^+, the mixture was treated with either 1.0 ml of 0.1 mol/l HCl (extracting NAD^+) or 1.0 ml of 0.1 mol/l NaOH (extracting NADH, destroying NAD^+). After incubation at 100 °C for 10 min, the mixture was centrifuged for 10 min at 10,000×*g* to collect the supernatant and stored at −21 °C. The pH relative to the collected supernatant was adjusted to 6.8 with HCl or NaOH (0.1 mol/l) before HPLC column application. A mixture of 90% H_3PO_4 (pH 6.6) and 10% methanol was used as mobile phase at a flow rate of 1.0 ml/min. The wavelength of the UV detector was set at 254 nm and the column temperature was controlled at 25 °C.

Results and Discussion

Effects of Xylose with Different Concentrations on Cell Growth and 1,3-PD in Shake Flask

The biomass and metabolites produced in response to xylose with different concentrations for culture 30 h were investigated (shown in Fig. 1). A large increase in biomass was obtained with xylose added by different concentration. As more xylose was added, more

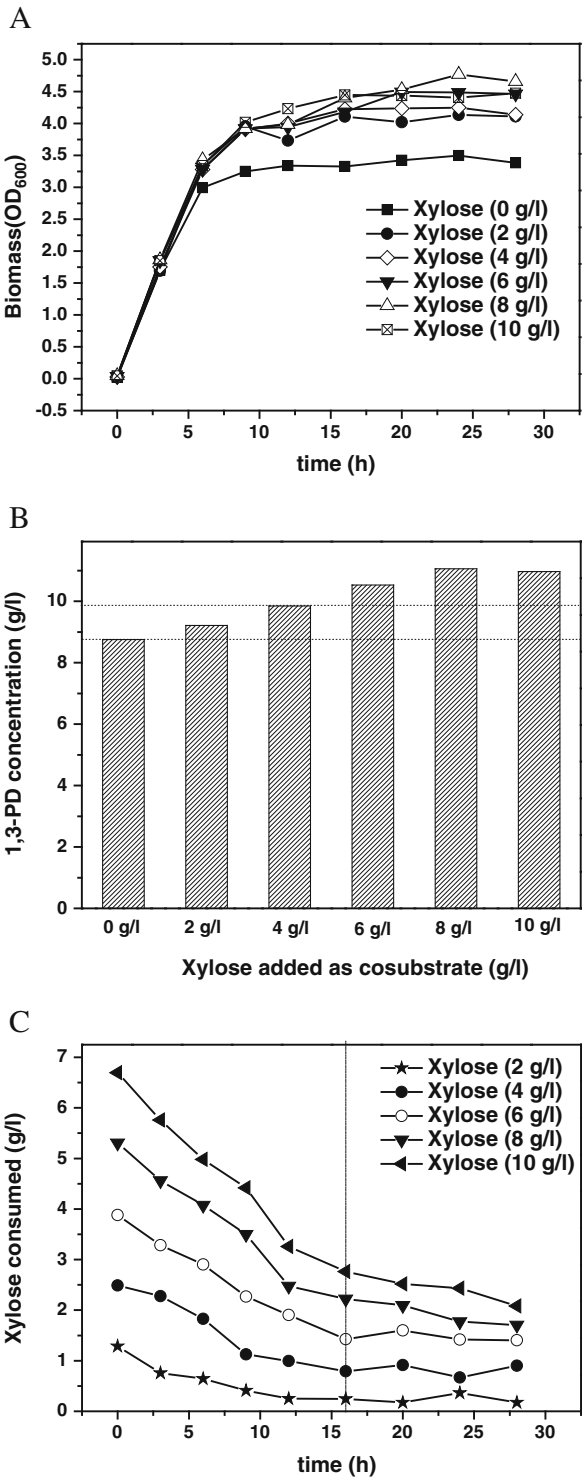

Fig. 1 Effects of xylose with different concentrations on cell growth and 1,3-PD production. All the data are the average of three experiments. **a** The effects of xylose with different concentrations on cell growth. **b** The effects of xylose with different concentrations on 1,3-PD production. **c** The residual xylose in medium was relevant to the initial concentration of xylose

biomass was obtained (Fig. 1a). The final biomass with 8 g/l xylose addition was the maximum. The effect of xylose with different concentrations on 1,3-PD production was similar with that on cell growth. As more xylose was added, more 1,3-PD was produced whereas the highest level (11.06 g/l) was reached at 8 g/l xylose which was 26.4% higher than that from glycerol alone. As indicated in Fig. 1c, xylose was utilized rapidly by *K. pneumoniae* during 0–16 h and was consumed little after 16 h, regardless of the variable concentration of xylose. It is worthy to point out that the residual xylose in medium was relevant to the initial concentration of xylose added in that as more xylose was added, more residual xylose was presented in the medium.

Adding sugar as cosubstrate was a feasible way to improve the 1,3-PD conversion and has been widely used [17–19]. Although the sugar could not be converted to 1,3-PD, it may be used for cell growth and regeneration of reducing power. The cofermentation of glycerol and xylose in 1,3-PD production by shake flask has been reported [20]. The 1,3-PD concentration and yield of 1,3-PD from glycerol with xylose addition in recombinant *Escherichia coli* were increased from 0.67 g/l to 1.08 g/l and from 0.46 mol/mol to 0.55 mol/mol, respectively. They considered that the availability of reducing power was one of the factors limiting the yield of 1,3-PD from glycerol, and the xylose catabolism could generate more reducing power for 1,3-PD pathway. However, the difference of physiological character between *K. pneumoniae* and *E. coli* was obvious. Rather, there was no related research work of mixture of glycerol and xylose in 1,3-PD production by *K. pneumoniae* or other bacteria with large application. If such a process of 1,3-PD production by using xylose as cosubstrate can be carried out, it will be contributed to find an outlet for the large application of hemicellulosic hydrolysates which contained xylose as the most abundant degradation contents.

1,3-PD Production with Xylose as Cosubstrate in Batch Fermentation by *K. pneumoniae*

The effects of xylose on batch fermentation by *K. pneumoniae* are shown in Table 1. The fermentation efficiency for 1,3-PD was significantly improved by xylose addition. The 1,3-PD concentration, conversion, and productivity by xylose addition were 13.20 g/l and 0.54 mol/mol, which were 9.4% and 20.0% higher than that from glycerol alone, respectively. Furthermore, other byproducts such as 2,3-BD, acetic acid, ethanol, succinic acid, and lactic acid were all improved. Especially, the 2,3-BD was almost doubly produced. The results also showed that more biomass can be gained in cofermentation process with xylose addition, which is helpful in promoting 1,3-PD biosynthesis.

Table 1 Effects of xylose on batch fermentation by *K. pneumoniae*

Number	AGC (g/l)	AXC (g/l)	Product concentration (g/l)						Conversion (mol/mol)	Biomass
			PD	BD	Suc	Ace	Eth	Lac		OD_{600}
1	29.05	–	12.1	2.08	0.97	1.39	2.36	1.44	0.45	5.48
2	29.41	6.75	13.2	3.87	1.13	1.58	2.87	2.01	0.54	6.43

Lac lactate, *Eth* ethanol, *Ace* acetate, *BD* 2,3-butanediol, *Suc* succinate, *1* batch fermentation with glycerol alone, *2* cofermentation with the addition of xylose, *AGC* accumulative glycerol consumed, *AXC* accumulative xylose consumed

Data are the average of three experiments

In previous studies [3,4], a positive correlation between the biomass of *K. pneumoniae* and its 1,3-PD production was demonstrated, whereas with more biomass, higher productivity was gained. We gained similar results. The production of 2,3-BD was much more than the former result (Table 2). This might be due to three reasons. First, 2,3-BD fermentation was favorable at a low pH of 6.5 [18] and especially preferred to the condition without pH control [21]. These conditions were carried out most of the time during the batch fermentation in this work. Second, more substrates (glycerol and xylose) and NADH flowed to 2,3-BD pathway. Third, xylose could be converted to 2,3-butanediol directly by *K. pneumoniae* [1].

Effects of Xylose on Internal Reducing Equivalent in Batch Fermentation

The 1,3-PD synthesis plays a key role in NADH consumption to regulate the intracellular reducing equivalent balance of *K. pneumoniae* by Zeng et al. [8]. Endeavor to enhance NADH generation should be taken into consideration for improving 1,3-PD production. Two distinct strategies can be used to alter the $NADH^+$ pool and $NADH/NAD^+$ ratio by San et al. [12]. The first approach is based on the use of carbon sources with different oxidation states. The other approach is based on genetic manipulations of the host cell and gained pronounced progress. In this project, we focused on the former approach to increase the reducing equivalent concentration.

The internal redox state in batch fermentation was determined from 4 to 16 h in batch fermentation (shown in Fig. 2). In fermentations by both batch fermentation and cofermentation, the total reducing equivalent (total NADH pools) increased significantly during 4–8 h and retained the highest level at 8 h and gradually decreased after 8 h. The result of the variation of reducing equivalent was similar to the study by Xu et al. [10]. This could be due to, in one aspect, to rapid cell growth which produced more NADH during the exponential period. In another aspect, the flexible physiological mechanism must shift the flux distribution to maintain the internal redox state. However, the metabolite flux to balance intracellular reducing equivalent could not consume NADH in time during this period. As the xylose was added, the total reducing equivalent (total NADH pools) were increased significantly by 1–3 mg/g (CDW) compared with that from glycerol alone, except at around 16 h (Fig. 2a). In batch fermentation without the addition of xylose, the NADH required for 1,3-PD biosynthesis process is mainly regenerated from NAD^+ along with oxidation of glycerol. Obviously, the accumulation of NADH is not favorable to synthesizing cell materials. As the xylose was added, the utilization of xylose through the pentose phosphate pathway provided much more additional reducing equivalent than that

Table 2 Effects of xylose in fed-batch fermentation by *K. pneumoniae*

Number	AGC (g/l)	AXC (g/l)	Product concentration (g/l)						Conversion (mol/mol)	Productivity (g/l/h)	Biomass (OD_{600})
			PD	BD	Suc	Ace	Eth	Lac			
1	128.72	25.26	67.21	6.82	7.60	10.25	12.55	40.57	0.63	1.82	13.95
2	143.16	–	60.78	4.10	5.81	9.76	9.24	31.17	0.52	1.64	10.45

Lac lactate, *Eth* ethanol, *Ace* acetate, *BD* 2,3-butanediol, *Suc* succinate, *1* fed-batch fermentation with xylose as cosubstrate, *2* fermentation with glycerol alone, *AGC* accumulative glycerol consumed, *AXC* accumulative xylose consumed

Data are the average of three experiments

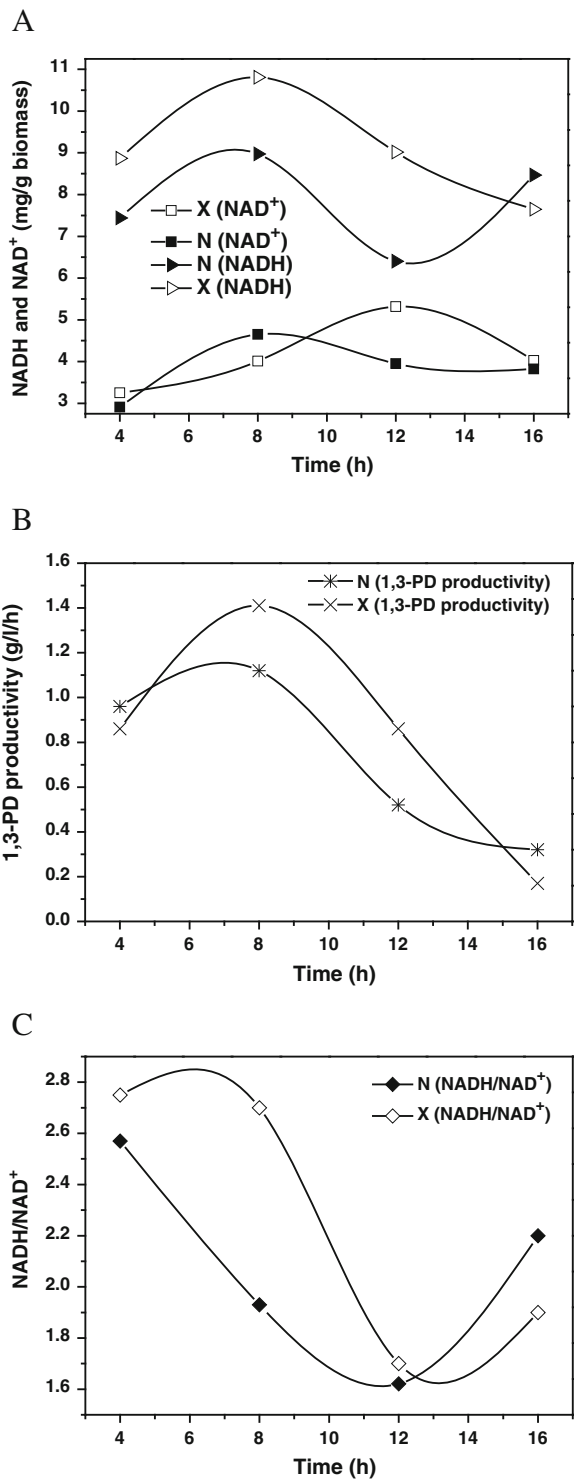

Fig. 2 Effects of xylose on internal reducing equivalent in batch fermentation. All the data are the average of three experiments. N (NAD^+), N (NADH), N (NADH/NAD^+), N (1,3-PD): all the data investigated in batch fermentation with glycerol alone; X (NAD^+), X (NADH), X (NADH/NAD^+), X (1,3-PD): all the data investigated in batch fermentation with xylose added. **a** Effects of xylose on internal reducing equivalent (NAD^+ and NADH). **b** Effects of xylose on 1,3-PD productivity. **c** Effects of xylose on the internal NADH/NAD^+ ratio

from glycerol alone. The variation of NADH was closely related with the fact that the variation of NAD^+ on purpose of balance of intracellular reducing equivalent. Interestingly, a significant increase in NAD^+ in batch fermentation with the addition of xylose was observed (Fig. 2a). The total NAD^+ in batch fermentation without xylose reached the highest level at 12 h and then gradually decreased while the highest level of NAD^+ from that of glycerol alone reached at 8 h (Fig. 2a).

1,3-PD production from glycerol is a model oxidoreduction-coupled biosynthesis process. The NADH/NAD^+ ratio which indicated that the internal redox state coincided with 1,3-PD production was observed in Fig. 2b, c. In batch fermentation without the addition of xylose, 1,3-PD productivity was increased gradually in exponential phase and consumed such a great number of NADH that aroused the decline of NADH/NAD^+ level. Undoubtedly, the NADH/NAD^+ level which dropped to a particular level would, in turn, limit 1,3-PD biosynthesis process. When the fermentation entered a stationary phase, the 1,3-PD productivity steadily declined and then led to an increase in its ratio. The NADH/NAD^+ ratio obtained higher levels by using xylose during 4–12 h, while the 1,3-PD productivity with the addition of xylose was much higher than that from glycerol alone (Fig. 2b, c). After 12 h, the NADH/NAD^+ ratio in the cofermentation was a little lower than that from glycerol alone. These variations of NADH/NAD^+ ratio of both fermentations were consistent with the time course of 1,3-PD productivity in batch fermentations. With the addition of xylose, 1,3-PD production during cofermentation was improved over that from glycerol alone from 6 to 12 h, while the increased reducing equivalent levels in cofermentation were higher than that from glycerol alone. The 1,3-PD productivity decreased rapidly after 8 h that kept pace with the variation of NADH/NAD^+ ratio. The result, as expected, also demonstrated that in cofactor-dependent production systems, cofactor availability and the proportion of cofactor in the active form may play an important role in dictating the overall process yield.

Effects of Xylose on Cell Growth and 1,3-PD Production in Fed-Batch Fermentation by *K. pneumoniae* under Microaerobic Conditions

For large-scale application of xylose and efficient production of 1,3-PD, fed-batch cultures of *K. pneumoniae* were performed with xylose as cosubstrate. In this study, we regulated the redox state variation online by the feeding rate (control the xylose 5–8 g/l and glycerol 15–20 g/l) during the fed-batch cofermentation. As indicated in Fig. 3a, xylose added as cosubstrate led to the rapid cell growth after 4 h. The final biomass (37 h) with xylose as cosubstrate reached 13.95 (OD_{600} nm) which was 25.1% higher than that from glycerol alone.

The effects of xylose on product formation are shown in Table 2 and Fig. 3. The indexes of 1,3-PD concentration, conversion, and productivity under microaerobic condition all improved substantially. With the addition of xylose, the 1,3-PD concentration, conversion from glycerol, and productivity increased respectively from 60.78 to 67.21 g/l, from 0.52 to 0.63 mol/mol, and from 1.64 to 1.82 g/l/h. The result was similar with that performed in batch fermentation except for 2,3-BD and lactate. The lower 2,3-BD production was performed in fed-batch fermentation since the pH controlled at 7.0 was unfavorable to 2,3-BD formation. However, the lactate formation was preferable to the pH control condition (pH 7.0), and more reducing power and substrates (glycerol and xylose) flowed to the lactate pathway.

Although the cofermentations of glycerol and other sugars were generally performed [17–19], the cosubstrates they used were still pure sugars, which inevitably resulted in high

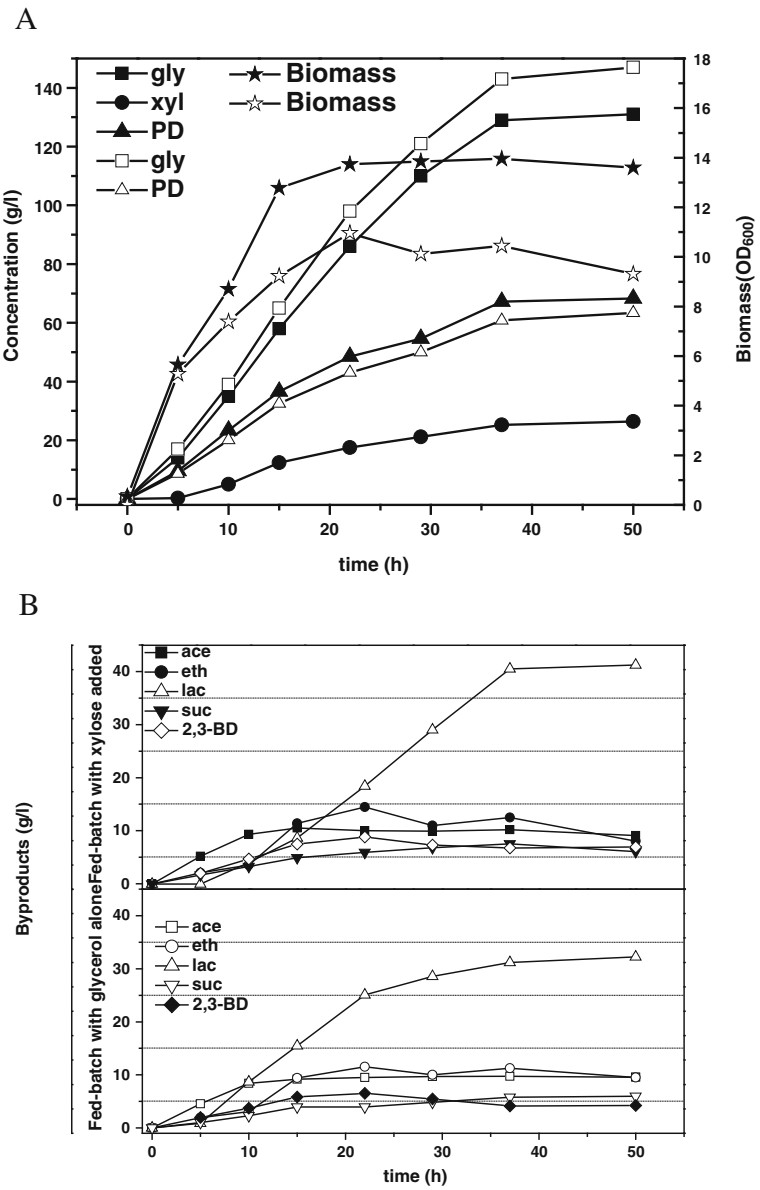

Fig. 3 Effects of xylose on cell growth and 1,3-PD production in fed-batch fermentation by *K. pneumoniae*. All the data are the average of three experiments. In (**a**), data with *filled symbols* were from cofermentation, and data with *unfilled symbols* were from fermentation with glycerol alone. *Lac* lactate, *Eth* ethanol, *Ace* acetate, *BD* 2,3-butanediol, *Suc* succinate, *PD* 1,3-propanediol, *Xyl* xylose consumed, *Gly* glycerol consumed. **a** Effects of xylose on cell growth, glycerol consumption, and 1,3-PD production during fed-batch fermentation. **b** Effects of xylose on byproducts during fed-batch fermentation

cost of 1,3-PD bio-production. However, xylose was the main carbohydrates derived from the hemicellulosic hydrolysates (HH). The related research works of HH added as cosubstrate in 1,3-PD production by *K. pneumoniae* are in progress in our laboratory.

Conclusion

The microbial production of 1,3-PD by *K. pneumoniae* was studied to demonstrate the feasibility of the fermentation using xylose as cosubstrate. The addition of xylose improved the 1,3-PD concentration from 12.07 to 13.20 g/l in batch fermentation. The reducing equivalent was increased significantly by 1–3 mg/g (CDW) compared with that from glycerol alone. With the addition of xylose in fed-batch culture, maintaining the xylose concentration at 5–8 g/l, 1,3-PD concentration, conversion, and productivity were respectively 10.58%, 21.11%, and 10.98% higher than that from glycerol alone. The biomass (OD_{600}) was increased significantly from 10.45 to 13.95.

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