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Full Length Research Paper

Production of 1,3-propanediol by *Klebsiella pneumoniae* using raw glycerol from *Zygosacharomyces rouxii*

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The conversion of glycerol to 1,3-propanediol (1,3-PD) is an environmentally friendly biological process and has attracted great interests worldwide. Efficient utilization of raw glycerol could bring significant economic benefits for 1,3-PD production. In the present study, the glycerol broth from strain *Zygosacharomyces rouxii* JL2011 was first used as the sole carbon source for 1,3-PD production by *Klebsiella pneumoniae*. The result indicates that the glycerol broth affected *K. pneumoniae* growth and 1,3-PD yield dramatically. Therefore, the raw glycerol was further prepared by extraction and purification, and the effect of raw glycerol on 1,3-PD production was investigated. The results obtained showed that the purified glycerol could be directly utilized in shake-flask culture by *K. pneumoniae* with a result comparable with that attained from pure glycerol. In addition, the fed-batch culture using raw glycerol as the substrate was conducted giving 56.1 $g \cdot L^{-1}$ with a yield of 0.40 $g \cdot g^{-1}$ glycerol and productivity of 1.12 $g \cdot L^{-1} \cdot h^{-1}$.

Key words: 1,3-propanediol, *Klebsiella pneumoniae*, *Zygosacharomyces rouxii*, raw glycerol.

INTRODUCTION

Glycerol is traditionally obtained as a by-product of fat hydrolysis in soap and other related materials production (Agarwal, 1990). Glycerol production has been widely studied by several microorganisms including *Candida* (Zhuge et al., 2001), *Pichia* (Hartlep et al., 2002) and *Zygosacharomyces* (Van et al., 1990). Also, as bio-diesel is produced by the transesterification of plant seed oils, the glycerol is the main by-product. Therefore, the supply of glycerol was significantly increased and surpluses the demand of the world market. So it is necessary to develop a new technology to convert glycerol into the other products of high value such as 1,3-propanediol (1,3-PD).

1,3-PD is one of the most remarkable raw materials for chemical industries due to its wide use in the different fields such as inks, polymers and medical applications.

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Annually, over one million ton of 1,3-PD is produced by chemical method, which requires high cost and is non-friendly to the environment. The microbial conversion of glycerol to 1,3-PD is particularly attractive because the process uses renewable feedstock and does not generate toxic byproducts (Biebl et al., 1999; Saxena et al., 2009). Several bacterial groups such as *Klebsiella* (Huang et al., 2002; Wang et al., 2011), *Citrobacter* (Boenigk et al., 1993) and *Clostridium* (Gungormusler et al., 2010; Saint-Amans et al., 2001) could convert glycerol to 1,3-PD in significant quantities. However, most researches have been conducted using pure glycerol as substrate. Nevertheless, a cost reduction in 1,3-PD production can be achieved by using less expensive substrates. Thus, it has been an economical and attractive route for 1,3-PD production using raw glycerol (Chatzifragkou et al., 2011; Gungormusler et al., 2011; Jun et al., 2010; Moon et al., 2010; Mu et al., 2006).

Zygosacharomyces rouxii JL2011 isolated from a high sugar environment can accumulate glycerol as the main osmolyte during osmotic stresses. It can convert up to

35% (w/w) of the available glucose into glycerol under optimum fermentation conditions, with glycerol accumulation up to 70 g/L in broth. The raw glycerol from *Z. rouxii* JL2011 may therefore be an interesting renewable carbon source for low-cost 1,3-PD production. In this study, we investigated the feasibility of 1,3-PD production by *Klebsiella pneumoniae* using different glycerol preparation derived from *Z. rouxii* JL2011 as the substrate.

MATERIALS AND METHODS

The glycerol broth from *Z. rouxii* JL2011 contained 60 to 70 g/L glycerol, 3.5 g/L acetate and 1.2 g/L ethanol. Pure glycerol (purity 99% w/w) and other chemicals were obtained commercially and of analytical grade.

Microorganism and media

Z. rouxii JL2011 and *K. pneumoniae* were stocked in our laboratory. The fermentation experiments with *Z. rouxii* JL2011 were carried out as described by Yagi et al. (1992). *K. pneumoniae* was used for 1,3-PD production from glycerol. The culture medium for *K. pneumoniae* consisted of the following per liter: 5 g yeast extract, 7.5 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.2 g $(NH_4)_2SO_4$, 0.005 g $FeSO_4 \cdot 7H_2O$, 1.5 µM coenzyme B_{12} and 5 ml trace element solution in each liter of media. The trace element solution contained (per liter): 0.68 g $ZnCl_2$, 2.0 g $MnCl_2 \cdot 4H_2O$, 60 mg H_3BO_3, 0.47 g $CoCl_2 \cdot 6H_2O$, 5 mg $Na_2MoO_4 \cdot 2H_2O$, 17 g $CuCl_2 \cdot 2H_2O$, 5.4 g $FeCl_3 \cdot 6H_2O$ and 10 ml HCl (37%). The diluted and sterilized supernatant from *Z. rouxii* JL2011 culture contained final concentration of 50 g/L glycerol at the beginning of fermentation. Five percent (v/v) of the seed culture was inoculated, and the initial pH of the medium was adjusted to 6.8. The fermentations were carried out in a rotary shaker incubator at 150 rpm and 30°C under aerobic conditions. The seed cultures were prepared in 250 ml flasks containing 50 ml LB medium, and incubated at 30°C for 18 h. Fermentation cultures were carried out in Erlenmeyer flasks (250 ml) containing 50 ml fermentation medium.

Fed-batch fermentation experiment

The seed cells for bioreactor were prepared in a 500 ml flask containing 100 ml seed culture. The flasks were incubated at 30°C for 24 h and subsequently inoculated into the fermenter at 6% (v/v). The fed-batch fermentation was conducted in 5-L fermenter (Corea Co. Ltd, KF-5L) containing 3 L fermentation medium under 0.3 vvm air flow. The pH was controlled at 6.8 by automatic addition of 3 M KOH and fermentation experiment was carried out at 30°C and 160 rpm. Glycerol was at 50 g/L and additional glycerol was added to maintain it between10 and 20 g/L during the fermentation. All assays were performed in duplicate; the reported values were the average from two assays.

Preparation of raw glycerol

The raw glycerol from glycerol was prepared using combined extraction and purification process according to the method described by Wang et al. (2001). The cell debris and other dissolved organic substances were removed by filtration and an inorganic inert material was added to the broth, then the glycerol broth was evaporated by distillation at 160 to 180°C. The raw glycerol (purity 87% w/w) was prepared from glycerol broth.

Analytical methods

Biomass concentration was estimated by measuring optical density (at 600 nm). Glucose was determined with immobilized glucose oxidase using a glucose analyzer. Glycerol concentration was confirmed with HPLC using an Aminex HPX 87H column (Bio-Rad) with a differential refractive index detector. As a mobile phase, 5 mmol/L H_2SO_4 (0.6 ml/min) was used at a working temperature of 50°C. Organic acid and ethanol were analyzed with an 1121-GS gas chromatography by using a 4 × 1 m stainless steel column. The determination of 1,3-propanediol was carried out with a gas chromatograph (SHIMAZU GC-14B, FID-detector, 2 m × Ø 5 mm stainless steel column packed with chromosorb101 and operated with N_2 as carrier gas at flow rate of 40 ml min^{-1}, detector temperature 220°C and column temperature 210°C).

RESULTS

Shake flask fermentation of 1,3-PD production by *K. pneumoniae* using different glycerol

To study whether glycerol broth from *Z. rouxii* JL2011 could be directly utilized in 1,3-PD production by *K. pneumonia*, the shake flask fermentation was performed. For comparison, 1,3-PD production was also carried out using pure glycerol. It took nearly 60 h to completely consume the glycerol in this culture. *K. pneumoniae* exhibited a long lag period of about 30 h for growth on the broth from *Z. rouxii* JL2011. Both the cell growth and 1,3-PD productivity was much lower in glycerol broth culture than those in pure glycerol culture (Figure 1). The different pattern of glycerol fermentation was observed compared to the normal shake-flask fermentation of pure glycerol. The final concentrations of 1,3-PD was 11.2 g L^{-1}. While in the control, the final concentration of 1,3-PD was 26.7 g L^{-1} (Table 1). This demonstrated that the glycerol broth from *Z. rouxii* JL2011 is not suitable to be used directly as a substrate to produce 1,3-PD production.

Moreover, after combining extraction and purification, the ethanol and acetate in glycerol broth were removed, thus forming the raw glycerol prepared. To examine the effect of the raw glycerol on the production of 1,3-PD, the culture of *K. pneumoniae* with pure glycerol and raw glycerol were conducted without pH regulation controlled with an initial pH of 6.8. Samples for product analysis were taken 5 h after inoculation. The results of fermentation experiments are shown in Figure 1. The conversion ratio of glycerol to 1,3-PD was 50.1%, which was similar to the results with pure glycerol (53.5%). No significant differences were observed concerning final product concentration and 1,3-PD yield (Table 1). This fact demonstrated that the raw glycerol had slight effect on batch cultures and was suitable for 1,3-PD production.

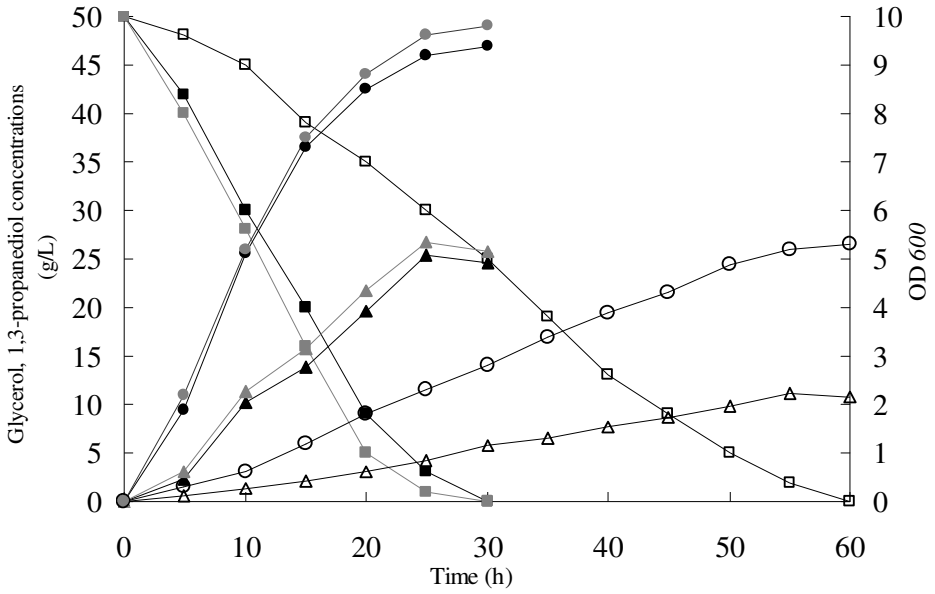

Figure 1. Time course of 1,3-PD batch fermentation by *K. pneumoniae* using different glycerol from *Z. rouxii* JL2011 (glycerol broth and raw glycerol) and pure glycerol as substrate, respectively. Glycerol broth as substrate: Biomass (○), residual glycerol concentration (□), 1,3-PD concentration (△); Raw glycerol as substrate: Biomass (●), residual glycerol concentration (■), 1,3-PD concentration (▲); Pure as substrate: Biomass (●), residual glycerol concentration (■), 1,3-PD concentration (▲). Values shown are averages of triplicate cultures with means less than 5 %.

Table 1. Comparison of products, residual glycerol, 1,3-propanediol (1,3-PD) in shake flask fermentation by *Klebsiella pneumoniae* with different glycerol from *Z. rouxii* JL2011 (glycerol broth and raw glycerol) and pure glycerol.

Type of glycerol	Initial glycerol concentration (g/L)	Products formed (g/L)			Conversion rate of glycerol to 1,3-PD % (w/w)
		Acetate	Ethanol	1,3-PD	
Glycerol broth	50	4.3 ± 0.1	1.76 ± 0.02	11.2 ± 0.1	22.4 ± 0.1
Raw glycerol		2.21 ± 0.02	0.74 ± 0.04	25.4 ± 0.2	50.1 ± 0.1
Pure glycerol		1.93 ± 0.05	0.55 ± 0.05	26.7 ± 0.3	53.5 ± 0.1

Fed-batch 1,3-PD production using raw glycerol as substrate

1,3-PD production was inhibited by a high glycerol concentration. To alleviate the inhibitory effect of high glycerol concentrations and enhance 1,3-PD yield, fed-batch supply of glycerol was evaluated. As shown in Figure 2 with the raw glycerol as substrate, the final concentration of 1,3-PD was 56.1 g L^{-1} with a yield of 0.40 g g^{-1} glycerol and productivity of 1.12 g L^{-1} h^{-1}.

DISCUSSION

In recent years, the utilization of glycerol to produce 1,3-PD has become increasingly attractive. The majority of the investigations have been conducted with pure glycerol employed as the sole carbon source. Since 50% of the entire cost of the microbial production of 1,3-PD is due to the price of raw materials, what seems to be important for an economically competitive fermentation process is the ability of *K. pneumoniae* to produce 1,3-PD in industrial processes.

In this study, the glycerol broth from the *Z. rouxii* JL2011 was used directly as substrate for 1,3-PD production by *K. pneumoniae,* which resulted in a low growth rate of *K. pneumoniae,* low glycerol consumption and many unfavorable products formed. The metabolites existing in the *Z. rouxii* JL2011 culture broth, such as acetate and ethanol, may interfere with the metabolism of *K. pneumoniae* (Table 1). It has been reported that acetate could significantly adversely affect the growth of *K. pneumoniae* and 1,3-PD production (Cheng et al., 2005; Hartlep et al., 2002). This could be one of the reasons for the observed low cell growth and 1,3-PD productivity in the shake-flask fermentation experiments. Fortunately, after a simple treatment with extraction and purification as previously described, the raw glycerol can

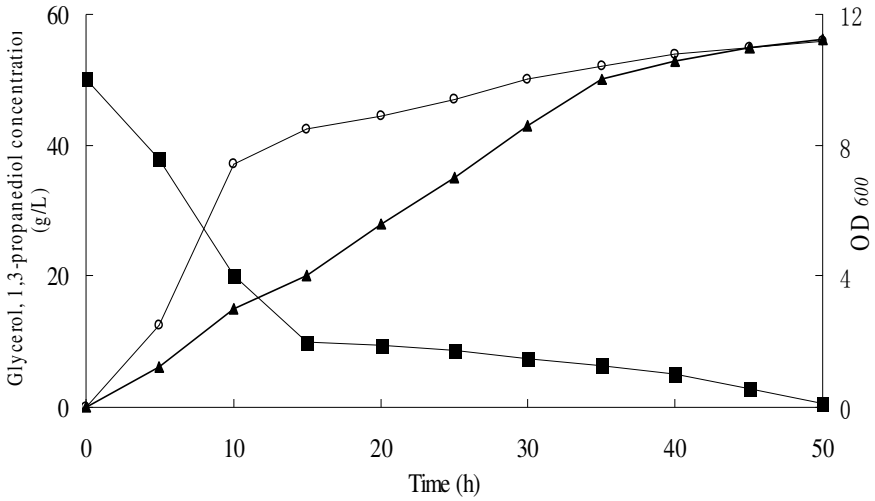

Figure 2. Time course of 1,3-PD fed-batch fermentation by *K. pneumoniae* using raw glycerol as substrate. Biomass (○), residual glycerol concentration (■), 1,3-PD concentration (▲). Values shown are averages of triplicate cultures with means less than 5%.

be used directly in batch culture of *K. pneumoniae* with results similar to those obtained from pure glycerol. Furthermore, in order to improve the yield of 1,3-PD and lessen the inhibitory effects of glycerol, the fed-batch culture using raw glycerol as the substrate was carried out, and the maximum 1,3-PD concentration achieved in fed-batch culture was 56.1 g L^{-1}.

This work therefore pave way for the production of 1,3-PD from low-cost feedstock, and may make a valuable contribution to the development of a cost-effective fermentation based on renewable resources. However, it is reported that 1,3-propanediol yield in anaerobic fermentation would be 0.53 g g^{-1} glycerol by control of metabolic flux (Zeng et al., 1993) and 0.43 g g^{-1} glycerol by anaerobic/aerobic combined fed-batch strategy (Cheng et al., 2004). Moreover, the 1,3-PD yield was only 0.4 g g^{-1} glycerol in this work, which was lower than the theoretical values. Hence, to further increase the yield of 1,3-PD, we attempt to combine metabolic engineering and different fed-batch strategy in a fermentor. Further works will be continued in our studies.

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