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# Enhance 1,3-propanediol production from crude glycerol in batch and fed-batch fermentation with two-phase pH-controlled strategy

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**Abstract** The batch fermentation of 1,3-propanediol (1,3-PD) by *Klebsiella pneumoniae* SU6 at different crude glycerol concentration (40-100 g $l^{-1}$), pH (6.5-7.5) and temperature (31-40ºC) combined with two-phase pH-controlled strategy was investigated. Effect of feeding rate (0.10-0.15 L $h^{-1}$) was studied in fed-batch fermentation. In batch fermentation, the optimal condition was 60 g $l^{-1}$ crude glycerol, pH control at 6.5 and cultivation temperature at 37ºC. The maximum 1,3-PD of 20 g $l^{-1}$, the yield of 0.34 g 1,3-PD $g^{-1}$ glycerol consumed and the productivity of 1.25 g $l^{-1}$ $h^{-1}$ were achieved at 16 hrs cultivation. The by-products were acetic acid and succinic acid at 2.7 and 1.1 g $l^{-1}$, respectively. Two-phase pH-controlled strategy gave better results (24.95 g $l^{-1}$ 1,3-PD and 1.78 g $l^{-1}$ $h^{-1}$ productivity) than constant pH-controlled strategy (20 g $l^{-1}$ and 1.25 g $l^{-1}$ $h^{-1}$, respectively) at 16 hrs incubation. In fed-batch fermentation, the maximum 1,3-PD of 45.35 g $l^{-1}$ was achieved at constant feeding rate of 0.1 L $h^{-1}$. The yield and productivity were 0.44 g $g^{-1}$ and 1.94 g $l^{-1}$ $h^{-1}$, respectively. The fed-batch fermentation with constant feeding at 0.1 L $h^{-1}$ with two-phase pH-controlled strategy gave 2.2 folds higher 1,3 PD concentration than the batch fermentation with two-phase pH-controlled strategy. This demonstrated the great impact of combination of pH control and feeding strategies in fed-batch fermentation on enhancing 1,3-propanediol production.

**Keywords:** 1,3 propanediol, crude glycerol, fed-batch fermentation, *Klebsiella pneumonia*, two-phase pH-control

## INTRODUCTION

Glycerol is a byproduct of biodiesel production (10 kg of biodiesel yields 1 kg of glycerol) (Yazdani and Gonzalez, 2007). The increase demand for biodiesel in the world market has increased the quantity of glycerol generated. Conversion of glycerol to high-value added commodity chemicals, especially 1,3-propanediol (1,3-PD) has received much attention in recent years. 1,3-PD can be used as a monomer to synthesize a new type of polyester, polytrimethylene terephthalate (PTT), which has excellent properties for use by textile and fiber industries (Zeng and Biebl, 2002). It also uses in the production of foods, lubricants, and medicines (Huang et al. 2002). Moreover, it can improve the properties of solvents (increase flexibility in blending ester quarts and other additives), adhesives, laminates, resins (low intrinsic viscosity, less solvent for coating), detergents (prevent phase separation and loss of enzyme activity), and cosmetics (long-lasting but not sticky moisturizing effect) (Liu et al. 2010). Production of 1,3-PD from glycerol using chemical method has several disadvantages, such as the requirement of high pressure and temperature for the chemical reaction, the use of toxic organic solvents, and gave low yields (5-15% w/w). Therefore, its microbial process is more interesting using several bacterial strains such as *Citrobacter freundii* (Homann et al. 1990), *Clostridium butyricum* (Papanikolaou et al. 2004), *Enterobacter agglomerans* (Barbirato et al. 1997) and *Klebsiella*

*pneumoniae* (Chen et al. 2003; Cheng et al. 2004; Xiu et al. 2004; Mu et al. 2008; Xue et al. 2010), *Klebsiella oxytoca* (Yang et al. 2007). Among these microorganisms, *K. pneumoniae* is of particular interest due to its flexible regulation of the carbon and reducing equivalent fluxes under different conditions (Zeng et al. 1993) as well as its higher product yield and productivity compared to other strains (Hao et al. 2008). In *K. pneumoniae*, glycerol is first converted to 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B12-dependent glycerol dehydratase (DhaB), which is then reduced to 1,3-PD by a reduced nicotinamide adenine dinucleotide (NADH)-dependent 1,3-PD oxidoreductase (DhaT) (Hong et al. 2010).

However, there are limitations of microbial synthesis like limited yields, titers and productivities, difficulties in the product separation from the medium, or the need of pretreatment of most of the raw substrates (Celińska, 2010). Several strategies have been developed to increase the concentration, yield and productivity of 1,3-PD especially the fed-batch process in which the addition of substrate is controlled to achieve a high cell density and high product formation by avoiding substrate inhibition (Huang et al. 2002; Mu et al. 2006; Zhang et al. 2007; Jun et al. 2009; Zhang et al. 2009; Xue et al. 2010). Although there are numerous reports demonstrating a high production of 1,3-PD using fed-batch fermentations, only few reports have evaluated the feasibility of using crude glycerol from biodiesel plant as a substrate for 1,3-PD production from *Klebsiella* species. In general, for 1,3-PD production, the optimal conditions used for cell growth and 1,3-PD production may be different depend on medium composition, environmental parameters, and type of cultivation.

Therefore, this work aims to evaluate the effects of glycerol concentration, pH and temperature combined with two-phase fermentation by controlled-pH strategy on cell growth and 1,3-PD production by *K. pneumoniae* SU6 in batch and fed-batch fermentation. To our knowledge, this is the first report on production of 1,3-PD using crude glycerol from biodiesel plant directly in fed-batch fermentation combined with two-phase pH-control.

## MATERIALS AND METHODS

### Microorganism

*Klebsiella pneumoniae* SU6 was isolated from domestic wastewater in Songkhla Province, Southern Thailand (Sattayasamitsathit et al. 2011) and has deposited at BIOTEC Culture Collection (BCC) (BCC No. 24246). The strain was maintained on Tryptic soy agar (TSA) (Difco, Michigan, USA) slant at 4ºC and subcultured every two months.

### Crude glycerol

Crude glycerol was obtained from Biodiesel Production Pilot Plant (capacity of 1000 L $d^{-1}$) at Faculty of Engineering, Prince of Songkla University. Waste cooking vegetable oil was used as raw material and the process employed an alkali-catalyzed transesterification reaction. It was boiled for 5 min before used.

### Culture medium

Glycerol medium used for inoculum preparation was consisted of 20 g $l^{-1}$ pure glycerol (99.9%, J. T. Baker), 3.4 g $l^{-1}$ $K_2HPO_4$, 1.3 g $l^{-1}$ $KH_2PO_4$, 2.0 g $l^{-1}$ $(NH_4)_2SO_4$, 0.2 g $l^{-1}$ $MgSO_4.7H_2O$, 1.0 g $l^{-1}$ yeast extract, 0.005 g $l^{-1}$ $FeSO_4.7H_2O$, 0.02 g $l^{-1}$ $CaCl_2$ and 2 ml trace element solution with the initial pH adjusted to 7.0 using 2N NaOH/HCl. Trace element solution contained 0.07 g $l^{-1}$ $ZnCl_2$, 0.1 g $l^{-1}$ $MnCl_2.4H_2O$, 0.06 g $l^{-1}$ $H_3BO_3$, 0.2 g $l^{-1}$ $CoCl_2.6H_2O$, 0.02 g $l^{-1}$ $CuCl_2.2H_2O$, 0.025 g $l^{-1}$ $NiCl_2.6H_2O$ and 0.035 g $l^{-1}$ $Na_2MoO_4.2H_2O$ (Hongwen et al. 2005).

Fermentation medium was consisted of 55 g $l^{-1}$ crude glycerol, 1.25 g $l^{-1}$ yeast extract, 5.5 g $l^{-1}$ ammonium phosphate and 2 ml $l^{-1}$ trace element solution (Sattayasamitsathit et al. 2011).

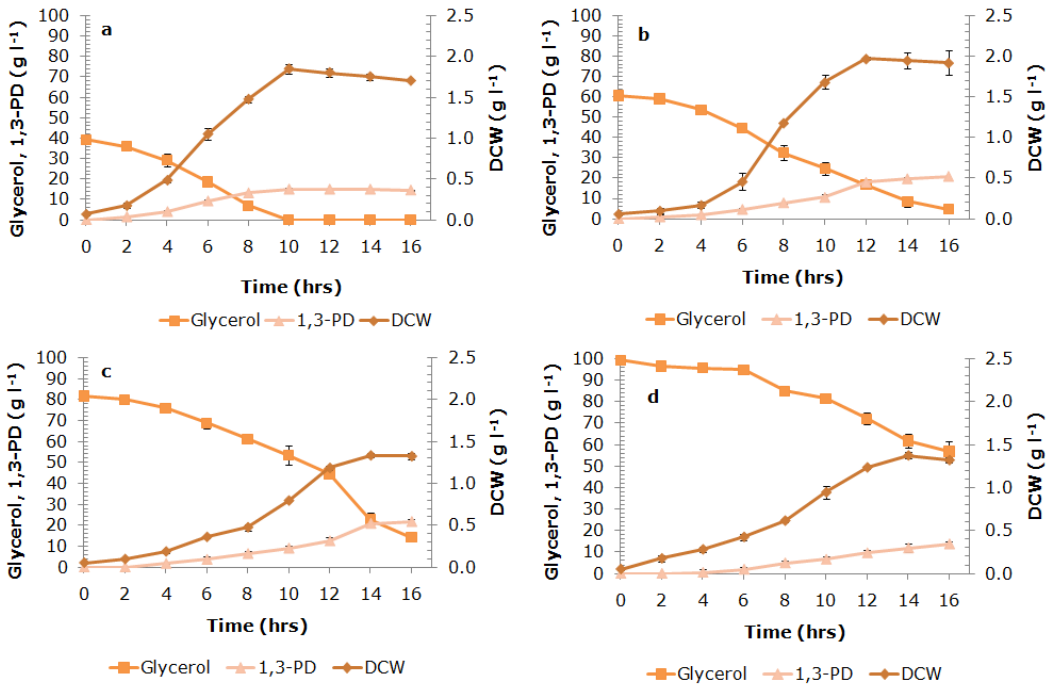


**Fig. 1 Time course of cell growth and 1,3-propanediol production by *K. pneumoniae* SU6 at different initial glycerol concentrations under controlled-pH 6.5 at 37ºC: (a) 40 g $l^{-1}$; (b) 60 g $l^{-1}$; (c) 80 g $l^{-1}$; (d) 100 g $l^{-1}$.**

### Batch fermentation

Inoculum for fermentation experiments was prepared by inoculating one loopful of slant culture into 200 ml glycerol medium. The seed culture was grown on a rotary shaker (200 rpm) at 37ºC for 24 hrs. The culture was diluted with sterile glycerol medium to obtain $OD_{600}$ of 0.5 before inoculating at 10%. Batch fermentation was performed in a 5L stirred-tank fermentor (B.E. Marubishi, Japan) at a working volume of 4L and 10% of seed culture was inoculated. Agitation was applied at a low rate just sufficient to mix the pH-control reagent but minimize mixing air into the medium (150 rpm). Micro-aerobic condition in the fermentor was applied by no air sparging. The pH was controlled at 6.5 using 5M KOH.

Optimization studies were conducted on various initial crude glycerol concentrations (40, 60, 80, 100 g $l^{-1}$), pH control (at 6.5, 7.0 and 7.5) and temperatures (31, 34, 37 and 40ºC) combined with two-phase pH-control. For two-phase pH-controlled strategy, pH was controlled at 7.5 at the first 6 hrs and then changed to 6.5 within 15 min after 6 hrs, other conditions were the same as those of constant pH experiment. Samples were taken to measure for dry cell weight (DCW), glycerol concentration, 1,3-PD and by-products (acetic acid and succinic acid). All fermentation assays were performed in triplicate and the presented results were the average values.

### Fermentative parameters

The fermentation parameters evaluated in this study were the yield of 1,3-propanediol defined as the ratio of 1,3-propanediol produced to glycerol consumed (g $g^{-1}$). The 1,3-propanediol productivity (g $l^{-1}$ $h^{-1}$) was calculated from the ratio of 1,3-propanediol concentration (g $l^{-1}$) to the fermentation time (hr). The cell yield per substrate consumed (g cell $g^{-1}$ glycerol), defined as the ratio of cell mass in the medium to the glycerol consumed (g $l^{-1}$) (Mussatto et al. 2008). The specific cell growth rate ($h^{-1}$) was determined from the slope of the semilogarithmic plot of cell density versus fermentation time (Meiying et al. 2002).

### Fed-batch fermentation

Fed-batch fermentation of *K. pneumoniae* SU6 were performed in 5L stirred-tank fermentors containing 2L of the fermentation medium at 37ºC and 150 rpm agitation speed. pH was controlled using two-phase strategies at pH 7.5 until glycerol concentration was lower than 15-25 g $l^{-1}$ (Xue et al. 2010), then changed to control at pH 6.5 using 5M KOH. The optimum initial glycerol concentration from batch culture was used in this study. After glycerol in the culture broth was lower than 15-25 g $l^{-1}$, then crude glycerol of 190 g $l^{-1}$ was fed to the culture broth at constant rates of 0.10 and 0.15 L $h^{-1}$ till the total volume reached 4L (full working volume). Samples of culture broth were taken at time interval to estimate the dry cell weight (DCW), glycerol and 1,3-PD concentration.

### Analytical methods

**Determination of dry cell weight (DCW).** Cell growth was determined as dry cell weight (DCW) by centrifugation the culture broth at 8,500 x *g* for 15 min at 4ºC and the washed cell sample was dried at 105ºC overnight and then weighed.

**Determination of glycerol, 1,3-propanediol and organic acids.** Samples taken during the fermentation runs were centrifuged at 8,500 x g for 15 min. The resulting supernatant was used for measurement of glycerol, 1,3-PD, acetic acid and succinic acid concentrations by High Performance Liquid Chromatography (HPLC) (Agilent 1200) equipped with a Bio-Rad HPX-87H (300mm x 7.8mm) column (Hercules, CA, USA) and a refractive index detector. The samples were diluted with deionized water, filtered through 0.22 µm, 13 mm Nylon membrane filter (Sartorius, German) and then injected in the chromatograph under the conditions: column temperature at 65ºC, 5 mM sulfuric acid as mobile phase at a flow rate of 0.7 ml $min^{-1}$, and an injection volume of 20 µL (Zhang et al. 2007). The concentration of these compounds was calculated using calibration curves obtained from standard solutions.

## RESULTS AND DISCUSSION

### Batch fermentation

**Effect of crude glycerol concentration.** Glycerol is a carbon and energy source for 1,3-PD production and its availability has an influence on the product formation (Zhang et al. 2007). Crude glycerol from biodiesel production contains not only glycerol but also impurities such as soap, methanol and free fatty acids. Its composition varies according to different oil feedstock and biodiesel production processes. Since the crude glycerol used in this study was obtained from biodiesel plant that used waste cooking oil as feedstock, therefore, its composition was first analyzed and found to contain only 27% glycerol and the impurities were methanol (1-3%), non-glycerol organic matter (1.6-7.5%), potassium and sodium salts (4-5%) and water (58%). It had a dark brown viscous liquid with a high pH level (11-12). Then, the influence of glycerol and impurities concentrations on growth and production of 1,3-PD in the batch culture of *K. pneumoniae* SU6 were tested. The effect of four levels of the initial glycerol concentration under controlled-pH at 6.5 at 37ºC is shown in Figure 1. The cell growth (1.92 g $l^{-1}$ DCW) was highest at 60 g $l^{-1}$ glycerol, while the highest 1,3-PD production at 60 and 80 g $l^{-1}$ glycerol were not significantly difference (21.50 and 21.63 g $l^{-1}$, respectively). At 10 hrs cultivation, 40 g $l^{-1}$ glycerol was completely consumed (100%) whereas the consumption at 60, 80 and 100 g $l^{-1}$ glycerol decreased to 56.7%, 32.5% and 18.0%, respectively. This clearly indicated that the high concentration of glycerol could inhibit cell growth that may be resulted from higher concentration of certain impurities in the crude glycerol. The presence of higher concentration of toxic substances may interfere with cell division and consequently reduce the cell viability such as sodium and heavy metals ions (Gonzalez-Pajuelo et al. 2004), methanol, soaps (Asad-ur-Rehman et al. 2008), methyl/ethyl esters, unreacted fatty acids (Furusawa and Koyama, 2004), glycerides and other natural compounds, such as phenolic antioxidants (Jerzykiewicz et al. 2009). The fast consumption of 40 g $l^{-1}$ glycerol within 10 hrs resulted in the highest specific growth rate (0.36 $h^{-1}$) as well as cell yield (0.043 g cell $g^{-1}$ glycerol) (Table 1). However, the maximum 1,3-PD concentration (21.63 g $l^{-1}$) and productivity (1.35 g $l^{-1}h^{-1}$) increased with the increase in the initial glycerol concentration up to 80 g $l^{-1}$ which was not significantly difference to those at 60 g $l^{-1}$ (21.50 g $l^{-1}$ and 1.34 g $l^{-1}h^{-1}$, respectively) (Table 1). Therefore, the optimal glycerol concentration for 1,3-PD production was in the range of 60-80 g $l^{-1}$. This optimum value was higher than *Enterobacter agglomerans* (45 g $l^{-1}$ glycerol) (Barbirato et al. 1997) and *K. pneumonia* (20-40 g $l^{-1}$ glycerol) (Zheng et al. 2008). Glycerol at higher concentration than 45 g $l^{-1}$ glycerol caused the accumulation of 3-hydroxypropionaldehyde (3-HPA) which was a strong inhibitory compound for

growth of *E. agglomerans* and cease the fermentation before glycerol exhaustion (Barbirato et al. 1997). This singular phenomenon was observed in *K. pneumoniae* and *C. freundii* (Barbirato et al. 1996). For *K. pneumoniae* grown in batch culture at an initial glycerol concentration of 20-40 g $l^{-1}$, high 1,3-PD production might be due to the increase of 3-HPA accumulation (Zhang et al. 2007). However, too high concentration of 3-HPA would repress the glycerol uptake and cell growth.

**Table 1. Effect of glycerol concentration on fermentation parameters of cell growth and 1,3-propanediol production by *Klebsiella pneumoniae* SU6 cultivated under controlled-pH (6.5) at 37ºC for 16 hrs.**

| Initial glycerol concentration (g $l^{-1}$) | Specific growth rate ($h^{-1}$) | Cell yield (g cell $g^{-1}$glycerol) | Maximum 1,3-PD concentration (g $l^{-1}$) | Product yield (g 1,3-PD $g^{-1}$glycerol) | Productivity (g $l^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|
| 40 | 0.36 | 0.043 ± 0.004[a] | 14.91 ± 0.63[a] | 0.38 ± 0.02[a] | 1.49 ± 0.004[a] |
| 60 | 0.39 | 0.036 ± 0.003[b] | 21.50 ± 2.12[b] | 0.32 ± 0.01[b] | 1.34 ± 0.035[b] |
| 80 | 0.22 | 0.028 ± 0.002[c] | 21.63 ± 1.13[b] | 0.33 ± 0.01[b] | 1.35 ± 0.033[b] |
| 100 | 0.18 | 0.027 ± 0.002[c] | 13.80 ± 0.49[a] | 0.31 ± 0.01[b] | 0.86 ± 0.023[a] |

[a,b] Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05.

In this study, at the end of fermentation time (16 hrs) *K. pneumoniae* SU6 was able to consume glycerol nearly completely (93%) up to 60 g $l^{-1}$ and tolerate higher concentration of crude glycerol than other previously reported strains. For example, cell growth of *K. pneumonia* XJ-Li was favourable of 20 g $l^{-1}$ of pure glycerol, giving 1,3-PD concentration of 12 g $l^{-1}$ and molar yield of 0.75. High concentration of glycerol at 30-50 g $l^{-1}$ inhibited cell growth and 1,3-PD production (Zhang et al. 2007). *Clostridium butyricum* VPI 3266 were able to grow on two types of crude glycerol (65% and 92% w/v glycerol) without any prior purification and no inhibitory effect was observed within 20 g $l^{-1}$ of both types (Gonzalez-Pajuelo et al. 2004). In this study, *K. pneumonia* SU6 could tolerate crude glycerol higher than *K. pneumoniae* DSM 4799 and all *Clostridium* strains including *C. butyricum* DSM 15410 that had the lowest consumption of glycerol (6.3 g $l^{-1}$) (Moon et al. 2010). It is anticipated that *K. pneumoniae* SU6 may have different mechanism for regulation of glycerol transport.

**Effect of pH.** The pH is one of the main factors influencing growth and 1,3-propanediol production by fermentation process because the catalytic activity of the enzymes and the metabolic activity of the microorganisms depend on the extracellular pH (Pirt, 1975). The optimal pH for growth may be different from that for product formation. Therefore, the effects of pH-controlled in the range of 6.5-7.5 on growth and production of 1,3-PD production and organic acids were studied using the optimal glycerol concentration obtained (60 g $l^{-1}$) and results are given in Figure 2. Glycerol was rapidly consumed at all pH-controlled values and depleted completely around 14-16 hrs. The glycerol consumption rates increased with increasing initial pH values (Figure 2a), 3.5, 4.2 and 7.8 g $l^{-1}$ $h^{-1}$ at pH 6.5, 7.0 and 7.5, respectively. This was agreed with the report that *E. agglomerans* consumed more glycerol at higher pH value and exhaustion of glycerol at pH 8 occurred after only 35 hrs cultivation (Barbirato et al. 1998). At pH 7.5 the growth was higher than those at pH 7.0 and 6.5 and reached a maximum of 2.1 g $l^{-1}$DCW at 14 hrs, then declined thereafter (Figure 2b). However, the specific growth rates (0.27-0.31 $h^{-1}$) and cell yields (0.026-0.031 g $g^{-1}$) of controlling pH at 6.5-7.5 were not significantly difference (Table 2). During the fast growth phase, both 1,3-PD and by-products increased rapidly (Figure 2c and 2e), illustrating their growth-associated characteristics. The maximum 1,3-PD concentration at each pH control showed an optimal value of 20.00 g $l^{-1}$ at pH 6.5, and decreased by 7.5% and 20% at pH 7.0 and pH 7.5, respectively at 16 hrs. The highest productivity could be achieved at pH 6.5 (1.25 g $l^{-1}$ $h^{-1}$) while those obtained at pH 7.0 and 7.5 were not significantly difference. However, the optimal product yield was 0.37 g 1,3-PD $g^{-1}$ glycerol at pH 7.0 (Table 2). Besides the main product 1,3-PD, by-products such as succinic acid, acetic acid, etc were also generated. At pH 6.5, 7.0 and 7.5, succinic acid was produced at 1.1, 1.2 and 1.5 g $l^{-1}$, respectively, whereas acetic acid production was 2.7, 3.2 and 3.5 g $l^{-1}$, respectively at the end of cultivation (16 hrs). These results indicated that high pH (7.5) could enhance organic acid synthesis better than lower pH (6.5). This agreed with previous report that alkaline condition favoured the formation of organic acids with a corresponding decrease in the yield of 1,3-propanediol (Garg and Jain, 1995). Based on the analysis of the phenomenon, a self-protection mechanism in *K. pneumoniae* so called switching the metabolic

pathways responding to environmental pH changes was proposed. However, the dependence of growth on acetate formation can be attributed to ATP formation in the acetate formation pathway, and growth favours reactivation of inactivated glycerol dehydratase in 3-HPA production (Xue et al. 2010). In this pathway, acetic acid concentration over 7.6 g $l^{-1}$ caused a 50% reduction in the growth and no growth occurred when the acetate concentration exceeded 15 g $l^{-1}$ at pH 6.8. The inhibitory effect of acetate was due to its undissociated form (Zeng et al. 1994) and at this pH, the critical concentration of acetate corresponded to only 0.14 g undissociated acetic acid $l^{-1}$. The inhibitory effect of undissociated acetic acid on *K. pneumoniae* M5al was even more severe than that on *K. pneumoniae* DSM 2026, which was able to grow in conditions of up to 0.46 g undissociated acetic acid $l^{-1}$ (Zeng et al. 1994).

The highest 1,3-PD production from *K. pneumoniae* SU6 at pH 6.5 was different to those from other microorganisms, such as *K. pneumonia* XJ-Li (pH 8.0) (Zhang et al. 2007), *K. pneumonia* ATCC 700721 (pH 7.37) (Oh et al. 2008), Thermophilic AT1 (pH 5.8) (Wittlich et al. 2001) and *C. butyricum* (pH 7.0) (Reimann et al. 1998). From the results discussed earlier, the optimal pH for 1,3-PD production and cell growth rate was different in constant pH controlled process. Therefore, it was favourable to use a pH-shift controlled process or two-phase pH-controlled process instead of constant pH controlled process.

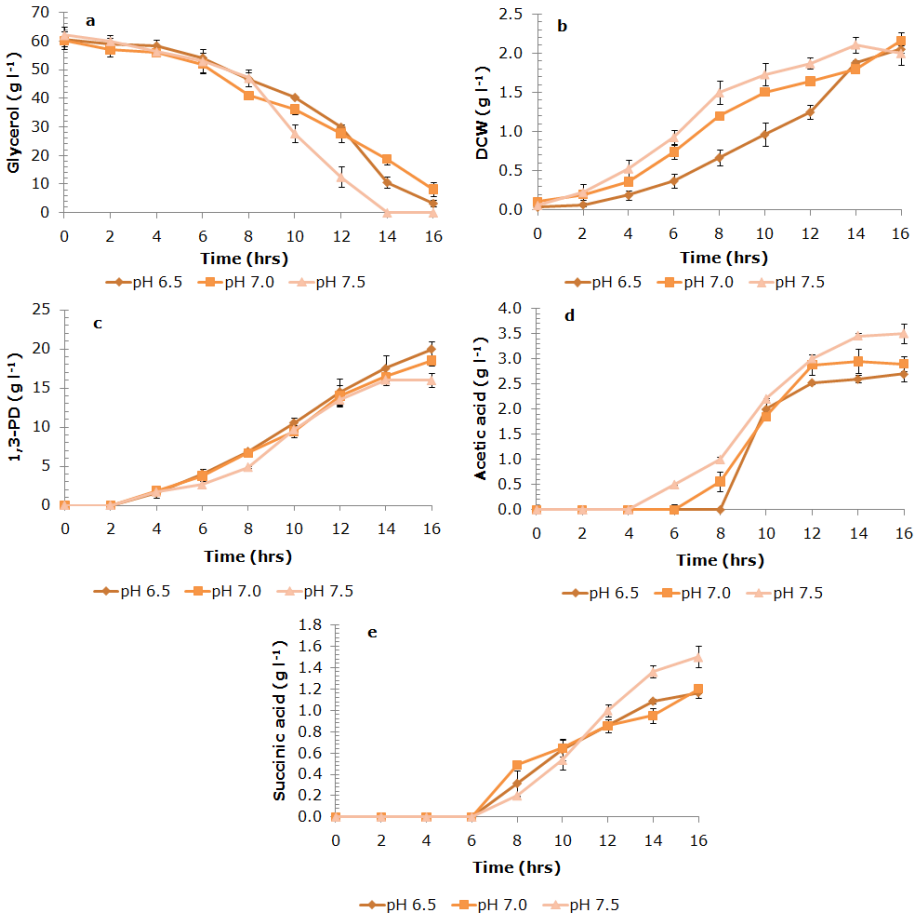


**Fig. 2 Time course of glycerol consumption, cell growth, 1,3-propanediol, acetic acid and succinic acid production by *K. pneumoniae* SU6 with initial glycerol concentration of 60 g $l^{-1}$ at 37ºC under different pH-controlled.**

**Table 2. Effect of pH control on fermentation parameters of cell growth and 1,3-propanediol production by *Klebsiella pneumoniae* SU6 with initial glycerol concentration of 60 g $l^{-1}$ cultivated at 37ºC for 16 hrs.**

| | Specific growth rate ($h^{-1}$) | Cell yield (g cell $g^{-1}$ glycerol) | Maximum 1,3-PD concentration (g $l^{-1}$) | Product yield (g 1,3-PD $g^{-1}$ glycerol) | Productivity (g $l^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|
| pH 6.5 | 0.27 | 0.031 ± 0.001[b] | 20.00 ± 1.70[a] | 0.34 ± 0.03[a] | 1.25 ± 0.08[a] |
| pH 7.0 | 0.30 | 0.026 ± 0.003[a] | 18.51 ± 1.13[a] | 0.37 ± 0.01[a] | 1.16 ± 0.06[a] |
| pH 7.5 | 0.31 | 0.027 ± 0.002[ab] | 16.18 ± 1.47[a] | 0.27 ± 0.03[b] | 1.00 ± 0.09[a] |

[a,b] Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05.

**Effect of temperature combines with two-phase pH-controlled condition.** As the pH needed for maximum cell growth and 1,3-PD production were different, a two-phase pH-controlled strategy was developed in which the pH was kept at 7.5 for the first 6 hrs (Figure 2b and 2c), and then switched to 6.5, to enhance the production of 1,3-PD. It combined with studying on the effect of temperature (31, 34, 37 and 40ºC). The results are shown in Figure 3 and summarized in Table 3. Cell growth in the temperature range of 31-37ºC showed no significantly difference in dry cell weight. But increase the temperature from 37ºC to 40ºC caused the 32-40% decrease on growth, cell yield, productivity and 1,3-PD production. The specific growth rate decreased nearly 75% (from 0.39 $h^{-1}$ to 0.10 $h^{-1}$). Therefore, above the optimum temperature (at 40ºC) inhibited cell growth due to the inactivation on the enzyme activity in glycolysis and the Krebs' cycle, then consequently decreased the carbon metabolism (Hochachka, 1968). The protein is temperature denatured by a reversible chemical reaction with free energy change and that denatured proteins of the enzymes are inactive (Roels, 1983).

The maximum concentration of 1,3-PD was 24.95 g $l^{-1}$ at 37ºC, but not a significantly difference in the range of 31-37ºC (24.07-24.95 g $l^{-1}$) except at 40ºC (16.87 g $l^{-1}$). This indicated a suitable temperature at 37ºC for the biosynthesis of 1,3-PD as well as the specific growth rate. Some essential enzymes and protein associated with cell growth and 1,3-PD production were inactivated or denatured when the temperature was too high. These results of *K. pneumoniae* SU6 were similar to those of *K. pneumoniae* (pH 7.0 and 30-37ºC) (Biebl, 2001) and *C. butyricum* (pH 7.0 at 35ºC) (Reimann et al. 1998). However, the product yields obtained from this study at all three temperatures (0.32 g $g^{-1}$) at 60 g $l^{-1}$ glycerol were much lower than those of *Citrobacter freundii* DSM 30039 (0.85 g $g^{-1}$), *K. pneumoniae* DSM 2026 (0.63 g $g^{-1}$) and *E. agglomerans* CNCM1210 (0.62 g $g^{-1}$) when using 20 g $l^{-1}$ glycerol (Homann et al. 1990). Therefore, *K. pneumoniae* SU6 could utilize high concentrations of glycerol that may have an important role in glycerol fermentation and convert to several by-products such as acetic acid (4.06 g $l^{-1}$) and succinic acid (1.7 g $l^{-1}$). This is an advantage because acetate could be responsible for the generation of growth energy, contributing 60% of the ATP production (Barbirato et al. 1995) and the yield of 1,3-PD is positively correlated with acetate (Zhang et al. 2006).

Comparison on glycerol fermentation at 37ºC between the two conditions revealed that two-phase pH-controlled strategy gave higher 1,3-PD and productivity (24.95 g $l^{-1}$ and 1.78 g $l^{-1}$ h, respectively) than those from constant pH-controlled process (pH 6.5) (20.00 g $l^{-1}$ and 1.25 g $l^{-1}$ h, respectively). 1,3-PD concentration and productivity could be increased by 19.84 and 29.78%, respectively. It is concluded that the proposed two-phase pH-controlled strategy can obviously enhance 1,3-PD fermentation.

**Table 3. Effect of temperature on fermentation parameters of cell growth and 1,3-propanediol production by *Klebsiella pneumoniae* SU6 with initial glycerol 60 g $l^{-1}$ cultivated under pH-controlledat 7.5 for 6 hrs, and switched to 6.5.**

| Temperature | Specific growth rate ($h^{-1}$) | Cell yield (g cell $g^{-1}$glycerol) | Maximum 1,3-PD concentration (g $l^{-1}$) | Product yield (g 1,3-PD $g^{-1}$ glycerol) | Productivity (g $l^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|
| 31ºC | 0.19 | 0.03 ± 0.002[a] | 24.07 ± 0.25[a] | 0.31 ± 0.01[a] | 1.72 ± 0.02[a] |
| 34ºC | 0.28 | 0.03 ± 0.003[a] | 24.93 ± 0.18[a] | 0.32 ± 0.01[a] | 1.78 ± 0.01[a] |
| 37ºC | 0.31 | 0.03 ± 0.002[a] | 24.95 ± 0.21[a] | 0.31 ± 0.03[a] | 1.78 ± 0.02[a] |
| 40ºC | 0.10 | 0.02 ± 0.001[a] | 16.87 ± 0.18[b] | 0.31 ± 0.02[a] | 1.21 ± 0.01[b] |

[a,b] Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05.

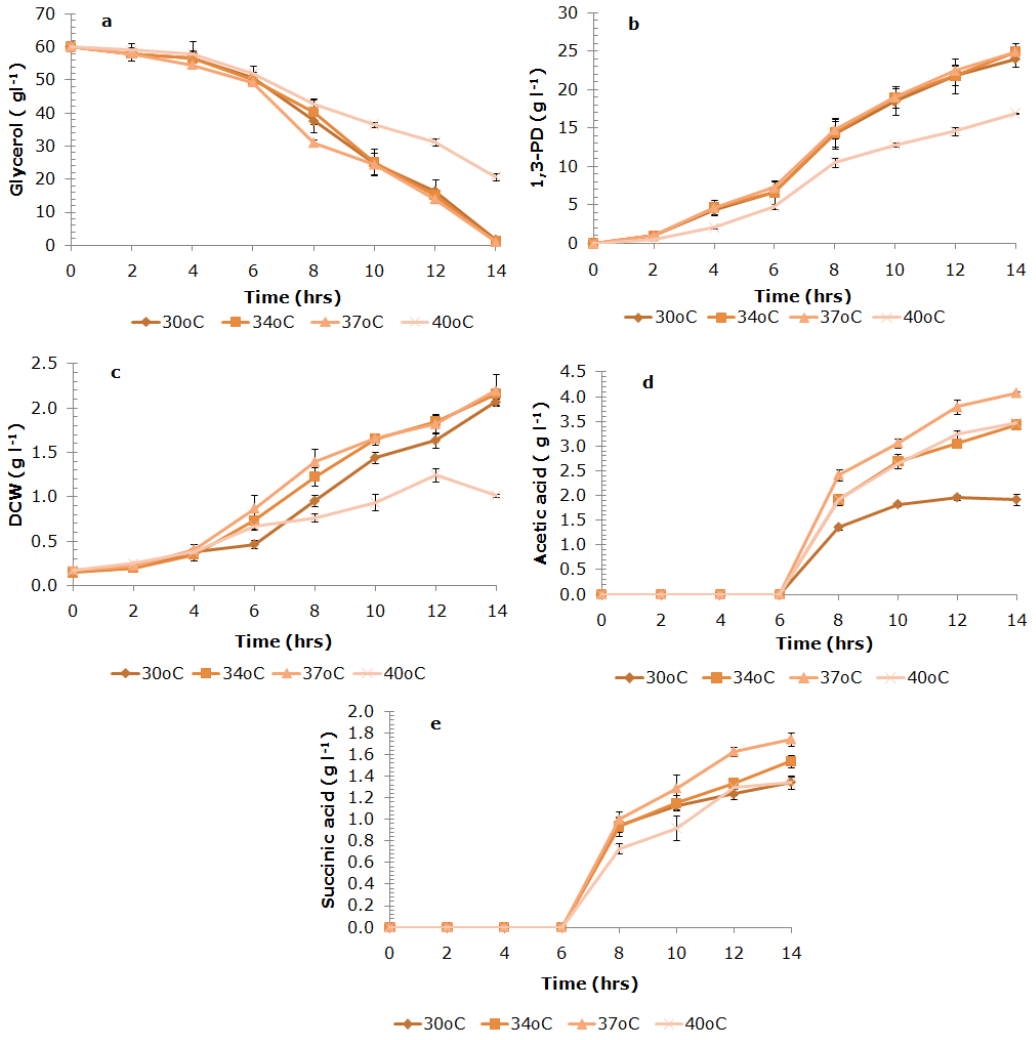


**Fig. 3 Time course of glycerol consumption, cell growth, 1,3-propanediol, acetic acid and succinic acid production by *K. pneumoniae* SU6 with initial glycerol concentration of 60 g $l^{-1}$ at 37ºC at different temperatures combined with two-phase pH-controlled condition from 7.5 to 6.5.**

## Fed-batch fermentation at different feeding rate with two-phase pH controlled condition on 1,3-PD production

Based on the results using batch fermentation, the accumulation of 1,3-PD is correlated to the increase of biomass. Since glycerol was exhausted at 14 hrs fermentation, no growth was observed thereafter. The production of biomass and 1,3-PD further increased as long as sufficient carbon source is available. Consequently, it is possible to enhance the yield of 1,3-PD by fed-batch fermentation. Fed-batch operation is frequently used for high cell density culture and, if cell growth is inhibited by excess nutrients, this mode of operation is very useful because the level of nutrients in the fermentor can be maintained at a low level (Chen et al. 2003). In order to examine the effect of feeding rate of crude glycerol with two-phase pH controlled condition on cell growth and 1,3-PD production, fed-batch operations were conducted with two different feeding rates of 190 g $l^{-1}$ glycerol at 0.10 and 0.15 L $h^{-1}$ (19 and 28.5 g glycerol $h^{-1}$, respectively). The fermentation was carried out in two phases. In the first phase, the microorganism was grown in batch culture under pH-control at 7.5 until glycerol was less than 25 g $l^{-1}$ (10 hrs). In the second phase, the medium was fed continuously into the fermentor at a constant feeding rate of 0.1 and 0.15 L $h^{-1}$ to a total volume of 4L and pH was controlled at 6.5.

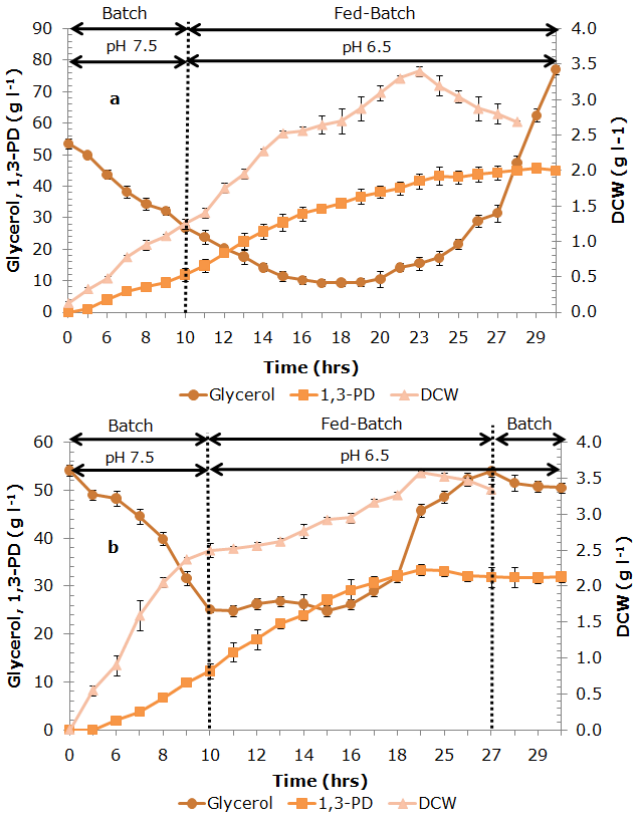


**Fig. 4 Time course of glycerol consumption, cell growth and 1,3-propanediol production at constant feeding fed-batch at 0.1 L $h^{-1}$ (a) and 0.15 L $h^{-1}$ (b) during cultivation of *K. pneumoniae* SU6 under two-phase pH-controlled condition from 7.5 to 6.5 at 37ºC.**

Figure 4 shows the results obtained during the cultivation of *K. pneumoniae* SU6 at constant feeding fed-batch 0.10-0.15 L $h^{-1}$ in a two-phase fermentation process. At the end of the first stage, about 1.2 g $l^{-1}$ DCW and 11.92 g $l^{-1}$ of 1,3-PD were produced. In the second stage (after 10 hrs cultivation), growth and 1,3-PD production increased rapidly in the 15-20 hrs cultivation period and stopped completely after 24 hrs. The maximum cumulative 1,3-propanediol concentration of 45.35 g $l^{-1}$ and the product yield of 0.44 g $g^{-1}$ (0.36 mol $mol^{-1}$) were achieved from the constant feeding rate at 0.1 L $h^{-1}$. The actual initial glycerol concentration was 53 g $l^{-1}$ (slightly lower than the expected concentration of 60 g $l^{-1}$). The productivity obtained was 1.75 g $l^{-1}$ $h^{-1}$ which similar to the previous result (1.78 g $l^{-1}$ $h^{-1}$). When increased the feed rate to 0.15 L $h^{-1}$, the maximum cumulative 1,3-propanediol concentration (33.25 g $l^{-1}$) decreased 1.36-fold compared to those from the feed rate at 0.1 L $h^{-1}$. The cumulative glycerol consumption decreased after 18 hrs cultivation due to the feed rate of glycerol was faster than its consumption rate. This resulted in the accumulation of glycerol concentration (70.54 g l-1) beyond the critical concentration (50 g $l^{-1}$) (Ji et al. 2009). Thus, the cells were in the stationary phase and hibernate quickly after 24 hrs and a few generation of 1,3-PD. Increasing the glycerol concentrations higher than 9-11% w/v did not allow efficient cell growth (Biebl, 1991). The 1,3-PD concentration (45.35 g $l^{-1}$) and productivity (1.75 g $l^{-1}$ $h^{-1}$) from *K. pneumoniae* SU6 were higher than those previously reported from *K. pneumoniae* (26.65 g $l^{-1}$ and 0.83 g $l^{-1}$ $h^{-1}$) (Ji et al. 2009), *K. pneumoniae* XJ-Li (38.10 g $l^{-1}$ and 0.79 g $l^{-1}$ $h^{-1}$) (Zhang et al. 2007) and *Klebsiella pneumoniae* M5al (41 g $l^{-1}$ and 0.72 g $l^{-1}$ h) (Cheng et al. 2006). However, 1,3-PD production from *K. pneumoniae* was improved by repeated fed-batch culture with addition of the organic acid mixture including citrate, fumarate and succinate (Xue et al. 2010). The fed-batch culture was repeated five times, and the 1,3-PD yield and concentration reached above 0.61 mol $mol^{-1}$ and 66 g $l^{-1}$, respectively, in 20 hrs for each cycle. Furthermore, the PDO productivity reached above 3.30 g $l^{-1}h^{-1}$ in each cycle, which was much higher than that of the original fed-batch culture.

It can be concluded that this fed-batch fermentation with two-phase pH controlled could enhance both 1,3-PD concentration and 1,3-PD productivity. The proposed fed-batch fermentation with two-phase pH controlled strategy was therefore proved to be successful approach to enhance 1,3-PD production.

## CONCLUDING REMARKS

Initial glycerol concentration, pH control and temperature had a profound effects on 1,3-PD fermentation by *K. pneumoniae* SU6. The optimum initial crude glycerol concentration was 60 g $l^{-1}$, giving the highest 1,3-PD concentration of 21 g $l^{-1}$, the product yield of 0.32 g $g^{-1}$ and productivity of 1.31 g $l^{-1}$ $h^{-1}$ at 16 hrs fermentation. Growth was maximum at pH 7.5 whereas pH 6.5 was optimum for 1,3-PD production. The optimum temperature combined with two-phase pH controlled strategy (shifting from 7.5 to 6.5) was superior for 1,3-PD fermentation to that of constant pH condition. The optimum temperature was in the range of 31-37ºC with the maximum cell yield of 0.030 g $g^{-1}$. The maximum concentration of 1,3-PD reached 24.95 g $l^{-1}$ with the product yield of 0.31 g $g^{-1}$ and the productivity of 1.78 g $l^{-1}$ h at 14 hrs fermentation. Two-stage pH-controlled strategy enhanced the conversion rate of glycerol to 1,3-PD and this led to an increment of productivity of 1,3-PD. Combination of these strategies in fed-batch fermentation exhibited the maximum concentration, yield, and productivity of 1,3-PD of 45.35 g $l^{-1}$, 0.44 g $g^{-1}$, and 1.94 g $l^{-1}$ $h^{-1}$, respectively, at constant feeding rate of 0.1 L $h^{-1}$ with actual glycerol concentration of 53 g $l^{-1}$. This gave 1.82 folds of 1,3 PD concentration compared with that from batch fermentation under the same condition.

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