

Full Length Research Paper

# Inhibitory mechanism of 3-hydroxypropionaldehyde accumulation in 1,3-propanediol synthesis with *Klebsiella pneumoniae*

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**3-Hydroxypropionaldehyde accumulation may cause the cessation of 1,3-propanediol sustained production with glycerol by *Klebsiella pneumoniae*. The impeller tip speed shift from higher to lower speed at glycerol excess or the pulsed glycerol feeding could lead to an abrupt increase of the 3-hydroxypropionaldehyde concentration (up to 10 mmol/l) in 10 min. The intracellular consequence of the 3-hydroxypropionaldehyde accumulation has not yet been elucidated. The rapid accumulation of 3-hydroxypropionaldehyde relying on the impeller tip speed shift was employed to investigate the influences of 3-hydroxypropionaldehyde to the activities of nine key enzymes related to glycerol metabolism, CO<sub>2</sub> and O<sub>2</sub> levels in off-gas, cell growth and 1,3-propanediol synthesis. Compared with that at 1.19 mmol/l 3-hydroxypropionaldehyde in broth, the residual enzymatic activities of the nine key enzymes ranged from 9.44 to 74.68% in the cultures at 7.5 mmol/l 3-hydroxypropionaldehyde in broth. The inhibitions of cell growth and the 1,3-propanediol synthesis were unnoticeable at the low level of 3-hydroxypropionaldehyde. By contrast, the CO<sub>2</sub> and O<sub>2</sub> levels changes in off-gas response to the 3-hydroxypropionaldehyde accumulation were less than 15 min. These results suggest that 3-hydroxypropionaldehyde inhibited the growth and metabolism of *K. pneumoniae* in a more complicated manner.**

**Keywords:** Fermentation, glycerol, 3-hydroxypropionaldehyde, *Klebsiella pneumoniae*, 1,3-propanediol.

## INTRODUCTION

Microbial production of 1,3-propanediol (1,3-PD) is one of the fast developing processes of bio-refinery, attracting attentions all over the world. The main application of 1,3-PD resides in the synthesis of polytrimethylene polyesters (PTT). The glycerol could be converted to 1,3-PD by bacterium belonging to the family of *Clostridia*, *Citrobacter*, *Lactobacillus* and also *Klebsiella* (Biebl and Marten, 1995; El-Ziney et al., 1998; Reimann et al., 1998). Glycerol dehydratase removes a water molecule from glycerol in the presence of coenzyme B<sub>12</sub> to form 3-hydroxypropionaldehyde (3-HPA). Subsequently, 1,3-PD

oxidoreductase transfers a reducing equivalent from NADH to 3-HPA, giving 1,3-PD. 3-HPA is the toxic intermediate metabolite, which could be used to protect food against microbial spoilage and also to produce acrylic acid (Vollenweider and Lacroix, 2004). However, the accumulation of 3-HPA cause the fermentation premature cessation at the excess of glycerol feeding (Barbirato et al., 1996).

In aqueous solution, the 3-HPA dimer is an analogue of D-ribose, which is able to inhibit the substrate binding subunit B<sub>1</sub> of the enzyme ribonucleotide reductase and thereby, inhibits the synthesis of DNA (Casas and Dobrogosz, 2000). Another toxicity effects is that 3-HPA induces oxidative stress in cells, most likely by modifying thiol groups in some proteins and small molecules (Schaefer et al., 2010). Other researchers focused on the inhibitory activity spectrum, environmental parameters

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**Table 1.** Effect of 3-HPA concentration on activities of key enzymes in pathway.

Enzyme	Enzymatic activity (U/mg)		Residue activities (%)
	ITS shift to 0.5 <sup>a</sup>	Without ITS shift	
	Con <sub>3-HPA</sub> =7.5 mmol/l	Con <sub>3-HPA</sub> =1.19 mmol/l	
Glycerol kinase	0.59±0.02	0.79±0.04	74.68±2.23
Glycerol dehydrogenase	0.046±0.002	0.48±0.002	9.58±0.43
Glycerol dehydratase	0.44±0.03	0.86±0.02	51.16±1.76
1,3-PD oxidoreductase	0.18±0.01	0.26±0.008	69.23±3.12
2,3-Butanediol oxidoreductase	0.19±0.01	0.28±0.01	67.86±2.89
Acetate kinase	0.31±0.02	1.55±0.07	62.00±2.03
Ethanol dehydrogenase	0.12±0.005	0.26±0.01	46.15±2.16
Lactate dehydrogenase	0.035±0.002	0.098±0.01	35.71±1.41
Fumarate reductase	0.0067	0.071±0.004	9.44±0.31

The batch cultures were conducted at initial 40 g/l glycerol, 37 °C and 0.65 vvm airflow. <sup>a</sup>ITS was shifted from 1.5 to 0.5 m/s at 9 h. The samples for the enzymatic detection were taken 10 min later.

influence and 3-HPA production optimization (Bauer et al., 2010; Cadieux et al., 2008; Tanaka et al., 2009). Different approaches including regulation of culture parameters and genetic modification were employed to prevent the lethal accumulation of 3-HPA and sustain the 1,3-PD fermentation in our practices ( Hao et al., 2008; Zheng et al., 2008a) .

However, the intrinsic physiological mechanism of 3-HPA action to *K. pneumoniae* has not yet been elucidated. The aim of this work was to investigate the influence of 3-HPA accumulation to the activities of key enzymes in the metabolic pathway, cell growth, CO<sub>2</sub> and O<sub>2</sub> levels in off-gas and 1,3-propanediol synthesis.

## MATERIALS AND METHODS

*K. pneumoniae* CGMCC 1.6366 was isolated from the soil of Tsinghua University campus and stored at the China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The basic culture medium contained glycerol (40 g), K<sub>2</sub>HPO<sub>4</sub> (0.69 g), KH<sub>2</sub>PO<sub>4</sub> (0.25 g), NH<sub>4</sub>Cl (4 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g), yeast extract (1.5 g) and 1 ml of trace element solution in 1 L water. The trace element solution was prepared as described previously (Zheng et al., 2010).

### Culture conditions

*K. pneumoniae* CGMCC 1.6366 was grown aerobically in 5 L B Braun Biostat B Plus (B. Braun, Germany) with a working volume of 4.0 L at initial 40 g/l glycerol, 37 °C and 0.65-vvm air flow. pH was kept constant at 6.5 by the automatic addition of 10 M NaOH. The batch cultures were conducted to investigate the enzymatic activities response to the 3-HPA accumulation. The impeller tip speed (ITS) was shifted from 1.5 to 0.5 m/s at 9 h. The samples for the enzymatic detection were taken 10 min later. The glycerol fed-batch cultures were used to investigate the cell growth, CO<sub>2</sub> and O<sub>2</sub> levels in off-gas and the 1,3-propanediol synthesis. In fed-batch experiments, the addition of pure glycerol into the broth started when the glycerol concentration in the broth was below 5 g/l. The glycerol level in the feeding cultures was regulated to 5 to 10 g/l. ITSs were shifted to 0.5 to 1 m/s at 8.5 and 10.5 h for 10 min to

induce 3-HPA accumulation in broth, respectively.

## Assays

Glycerol and 1, 3-PD were determined by a Shimadzu 10AVP HPLC system using Aminex HPX-87H column (300×7.8 mm) (Bio-Rad, Palo AHO, Ca, USA) as described (Zheng et al., 2008b). 3-HPA was determined using the colorimetric method (Circle, 1945). The CO<sub>2</sub> and O<sub>2</sub> concentrations in the off-gas were determined using a GXH-510 gas analyzer (Xibi, Beijing, China). Dry cell mass (biomass) was calculated from OD<sub>650</sub> with the optical density calibration curve.

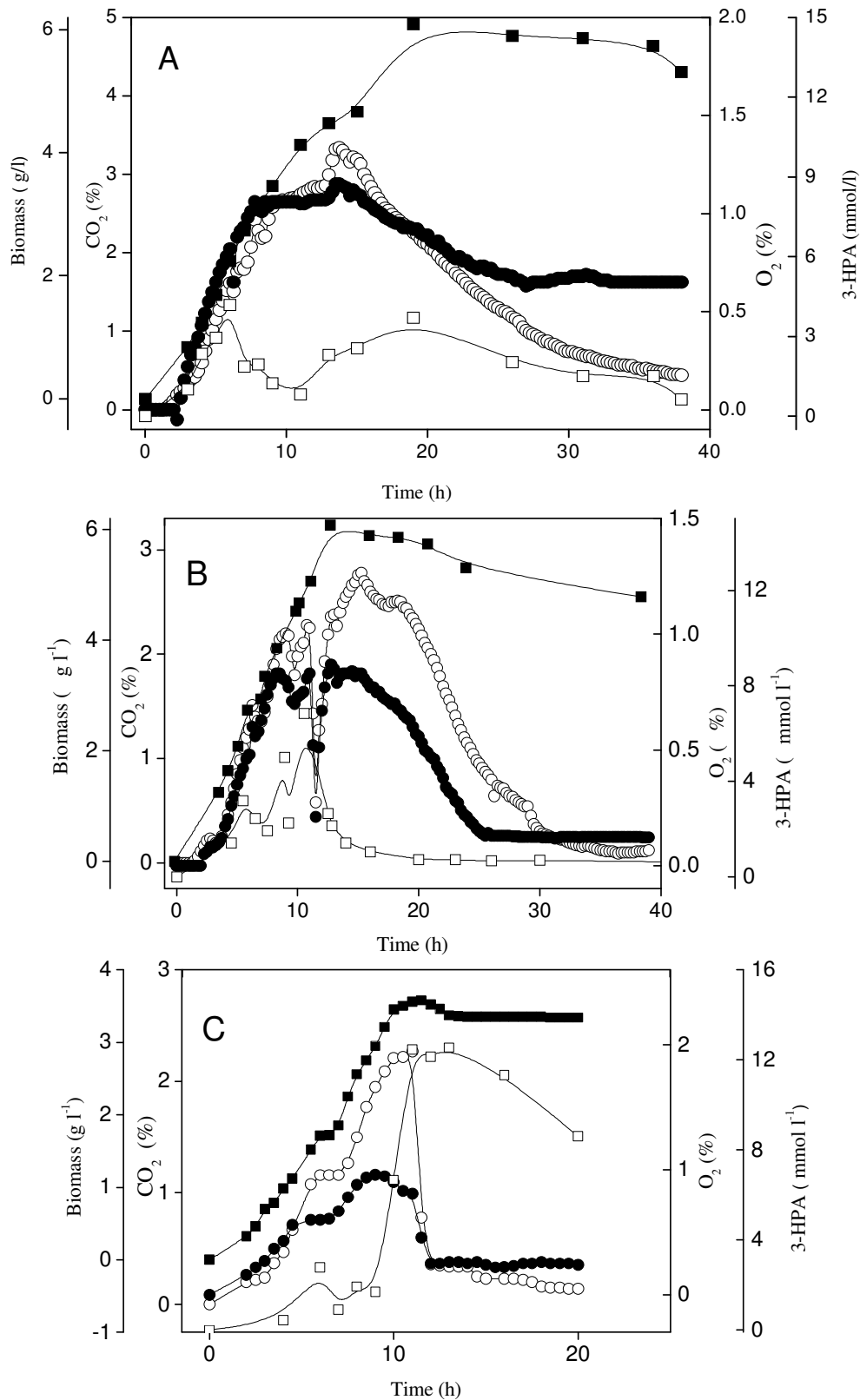
## Measurement of enzyme activities

The measurements of activities of glycerol dehydratase, glycerol kinase, glycerol dehydrogenase, 1,3-propanediol oxidoreductase, fumarate reductase, 2,3-butanediol oxidoreductase, lactate dehydrogenase, acetate kinase and ethanol dehydrogenase were described previously (Zheng et al., 2008b). One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of product per min.

## RESULTS AND DISCUSSION

### Influence of 3-HPA on key enzymes

ITS shift experiments were performed to determine the enzymatic activities response to 3-HPA accumulation. Nine enzymes in *K. pneumoniae* metabolic pathway including glycerol dehydratase, glycerol kinase, glycerol dehydrogenase, 1,3-propanediol oxidoreductase, fumarate reductase, 2,3-butanediol oxidoreductase, lactate dehydrogenase, acetate kinase and ethanol dehydrogenase were selected to detect the enzymatic activities. These enzymes are related to the glycerol uptake, 1,3-PD and other products synthesis. Compared with the fermentation at ITS of 1.5 m/s, levels of the earlier mentioned enzyme activities showed different residual value (Table 1) in the fermentation with ITS shift from



**Figure 1.** (A) Biomass, O<sub>2</sub> consumption and CO<sub>2</sub> synthesis profile in 1,3-PD fed-batch fermentation: biomass (closed squares), O<sub>2</sub> (closed circles), CO<sub>2</sub> (open circles), 3-HPA (open squares). The batch cultures were conducted at initial 40 g/l glycerol, 37°C and 0.65 vvm airflow; ITS was shifted to 1 and 0.75 m/s at 8.5 and 10.5 h for 10 min (B); to 0.5 m/s at 10.5 h for 10 min (C). There was no ITS shift in A.

**Table 2.** Final 1,3-propanediol concentrations from different ITS shift fed-batch fermentations.

Final 1,3-propanediol con. (g/l)	ITS shift (m/s)	
	8.5 h	10.5 h
68.74±3.52	-	-
69.23±4.41	1	0.75
12.99±0.37	-	0.5

ITSs were shifted to 0.5 to 1 m/s at 8.5 and 10.5 h for 10 min to induce 3-HPA accumulation, respectively.

1.5 to 0.5 m/s. Glycerol kinase retained the highest residual activity of 74.68%, whereas fumarate reductase had the lowest value of 9.44%. What should be noted is that glycerol dehydrogenase, 1,3-PD oxidoreductase, 2,3-butanediol oxidoreductase and ethanol dehydrogenase belong to the short-chain alcohol dehydrogenase (SCAD) superfamily, sharing the same conserved sequence motifs (Breitling et al., 2001; Qin et al., 2000). However, the residual activities of glycerol dehydrogenase, 1,3-PD oxidoreductase, 2,3-butanediol oxidoreductase and ethanol dehydrogenase were 46.15, 69.23, 67.86 and 51.16%, respectively. The explanation to this discrepancy could be that there is great diversity in the SDAR superfamily or 3-HPA inhibited the enzyme mentioned earlier in a more complicated manner other than the specific manner as previously stated.

### Influence of 3-HPA to cell growth, 1,3-PD production, CO<sub>2</sub> and O<sub>2</sub> metabolism

The lethal 3-HPA accumulation during 1,3-PD fermentation leads to the systematic inhibition of *K. pneumoniae*. The characteristic physiological property was that glycerol uptake, cell growth ceased, and pH value of the fermentation broth increased. In order to further the investigation of the consequences of 3-HPA accumulation, ITS shift was conducted in the glycerol-fed batch fermentation. The response of the cell growth, CO<sub>2</sub> and O<sub>2</sub> amount in off-gas to 3-HPA were monitored. The addition of pure glycerol into the fed-batch cultures was initiated when the glycerol concentration in the broth was below 5 g/l. Unlike biomass, O<sub>2</sub> consumption and CO<sub>2</sub> synthesis were more sensitive to high 3-HPA concentration. Compared with that in the control fermentation with low 3-HPA level (Figure 1a), O<sub>2</sub> and CO<sub>2</sub> levels in off-gas decreased from 2.25 and 0.83 to 1.43 and 0.52% within 15 min after the 3-HPA peak of 6.85 mmol/l, respectively (Figure 1b). The short response time to the variation of 3-HPA concentration was against the previous statement that indicated that 3-HPA directly inhibits cell growth via DNA synthesis (Vollenweider and Lacroix, 2004). It may inhibit the enzymes in the electron transfer chain or the central carbon pathway at the post translation level. 1,3-PD synthesis inhibition were not

noticeable at low level of 3-hydroxypropionaldehyde (Table 2). As expected, the fed-fermentation ceased due to the ITS shift which caused 12.45 mmol/l 3-HPA accumulation. O<sub>2</sub> consumption and CO<sub>2</sub> synthesis decreased to very low level (Figure 1c). The final 1,3-PD concentration was only 12.99 g/l due to the cessation of the fermentation (Table 2). Taken together, 3-HPA did not inhibit the enzyme activities in *K. pneumoniae* specifically. It appears that the screening of 3-HPA tolerant isolate is not a feasible solution to 3-HPA threat issue, instead, the online metabolite monitor may facilitate the prevention of the lethal accumulation of 3-HPA and realize the sustained 1,3-PD production. The inhibitory behaviors of 3-HPA accumulation relying on RT-PCR, Western blotting and analysis with isolated enzymes will be discussed in details in another paper.

### Conclusions

Lethal 3-HPA accumulation influenced the 1,3-PD production. High ITS may prevent the lethal 3-HPA accumulation at the expense of 1,3-PD yield. ITS shift was employed to investigate the consequence of 3-HPA accumulation in this work. The earlier mentioned selected enzymes retained the different residual activities at 7.5 mmol/l of 3-HPA. Although 1,3-PD oxidoreductase and glycerol dehydrogenase share the conserved motif of SCAD superfamily, 1,3-PD oxidoreductase had 1.5 times residual activity than that of glycerol dehydrogenase. The response time to the 3-HPA accumulation of CO<sub>2</sub> and O<sub>2</sub> levels in off-gas was only less than 15 min, suggesting that 3-HPA may not directly inhibit cell growth via DNA synthesis, but inhibit the enzymes in the electron transfer chain or the central carbon pathway at the post translation level. This work provides some insights in how 3-HPA inhibits cell growth and intracellular enzyme catalysis.

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