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ORIGINAL PAPER

3-Hydroxypropionaldehyde guided glycerol feeding strategy in aerobic 1,3-propanediol production by *Klebsiella pneumoniae*

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Abstract 3-Hydroxypropionaldehyde (3-HPA) is a toxic intermediary metabolite in the biological route of 1,3-propanediol biosynthesis from glycerol. 3-HPA accumulated in culture medium would arouse an irreversible cessation of the fermentation process. The role of substrate (glycerol) on 3-HPA accumulation in aerobic fermentation was investigated in this paper. 1,3-Propanediol oxidoreductase and glycerol dehydratase, two key enzyme catalyzing reactions of 3-HPA formation and consumption, were sensitive to high concentration of 3-HPA. When the concentration of 3-HPA increased to a higher level in medium (ac 10 mmol/L), the activity of 1,3-propanediol oxidoreductase in cell decreased correspondingly, which led to decrease of the 3-HPA conversion rate, then the 3-HPA concentration increasing was accelerated furthermore. 3-HPA accumulation in culture medium was triggered by this positive feedback mechanism. In the cell exponential growth phase, the reaction catalyzed by 1,3-propanediol oxidoreductase was the rate limiting step in 1,3-propanediol production. The level of 3-HPA in culture medium could be controlled by the substrate (glycerol) concentration, and lower level of glycerol could avoid 3-HPA accumulating to a high, lethal concentration. In fed batch fermentation, under the condition of initial glycerol concentration 30 g/L, and keeping glycerol concentration lower than 7–8 g/L in cell exponential growth phase, 3-HPA accumulation could not be incurred. Based on this result, a glycerol feeding strategy was set up in fed batch fermentation. Under the optimized condition, 50.1 g/L of 1,3-propanediol was produced in 24 h, and 73.1 g/L of final 1,3-propanediol concentration was obtained in 54 h.

Keywords 3-Hydroxypropionaldehyde · *Klebsiella pneumoniae* · 1,3-Propanediol · Feeding strategy

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Introduction

1,3-Propanediol is an important chemical mainly used for the synthesis of polytrimethylene terephthalate and other polyester fibers [28]. A variety of chemical routes for 1,3-propanediol production using acrolein or ethylene as substrate were known [4, 17]. 1,3-Propanediol could also be produced by biotechnological route, in which glycerol was converted to 1,3-propanediol by various species of prokaryote strains, such as *Klebsiella pneumoniae* [11], *Klebsiella oxytoca* [16], *Enterobacter agglomerans* [5], *Citrobacter freundii* [8], *Clostridium butyricum* [2] and *Clostridium pasteurianum* [7]. Among them, *K. pneumoniae* and *C. butyricum* were most popular investigated. Glycerol is utilized as substrate in biotechnological route, which is renewable and can be easily obtained from discharge of bio-diesel and soap manufacturing units [23]. It is expected that an over-capacity of more than 600,000 metric tones of glycerol residue would be produced from bio-diesel industry, based

on the growth of bio-diesel industry in Europe. Currently, crude glycerol water derived from bio-diesel plants in most Western Europe countries is treated as a typical ''industrial waste water'', which makes using glycerol as raw material for 1,3-propanediol production at a significantly low cost [22]. At the same time, the strong fluctuations of crude oil price increased the cost of chemical routes, so the biotechnological route appeared more attractive.

A 1,3-propanediol producing strain had been isolated by our research team, which belonged to the species of *K. pneumoniae subspecies Pneumoniae* and was named TUAC01 (*Kp*TUAC01). *Kp*TUAC01 had an outstanding 1,3-propanediol producing property in aerobic condition, which was different to other reported anaerobic 1,3-propanediol producing strains [14]. In our preliminary investigation of the strain, cessations were often occurred in the fermentation process. Barbirato had reported 3-HPA aroused 1,3-propanediol production cessation in anaerobic fermentation by *E. agglomerans* [5]. 3-HPA accumulation during the fermentation of glycerol to 1,3-propanediol by *K. pneumoniae* and *C. freundii* was also presented in his paper. By analyzing the fermentation broth, it was found those cassations occurred in *Kp*TUAC01 were also caused by the toxicity of 3-HPA. 3-HPA was an intermediary metabolite in the process of conversion glycerol to 1,3-propanediol (see Fig. 1). Likewise, Barbirato stated that 3-HPA accumulation depended on the ratio of glycerol dehydratase activity to 1,3-propanediol oxidoreductase activity [6]. In our previous research, a constructed strain was obtained by cloning and over expressing the 1,3-propanediol dehydrogenase gene in *Kp*TUAC01. In the constructed strain, a higher 1,3-propanediol oxidoreductase activity was detected, and the level of 3-HPA in culture medium was obviously decreased. But there was a drawback in the constructed strain, the productivity and the final 1,3-propanediol concentration were lower than the wild strain *Kp*TUAC01. The reason was the multi-copy plasmid in constructed strain affected cell growth [15].

In the biotechnological route of 1,3-propanediol production, the processes including batch fermentation, fed batch fermentation, continuous fermentation and immobilized cell fermentation were all reported. Some data of final 1,3-propanediol concentration, yield of glycerol to 1,3-propanediol and productivity were list in Table 1. Batch fermentation was simple and often used in primary investigation. Continuous fermentation and immobilized cell fermentation had higher productivity, but the final concentration of 1,3-propanediol was low. A modified two-stage continuous culture achieved relative higher final 1,3-propanediol concentration. Fed batch fermentation present higher final 1,3-propanediol concentration together with higher productivity. The down steam processes of 1,3-propanediol production is difficult, for this reason, higher final 1,3-propanediol concentration may have more advantages. So that, fed bath fermentation is usually selected for industrial purpose. In this paper, the effect of substrate (glycerol) on 3-HPA accumulation in aerobic 1,3-propanediol production was investigated. And a method of adjusting glycerol concentration in culture medium to alleviate 3-HPA accumulation was exploited in fed batch fermentation.

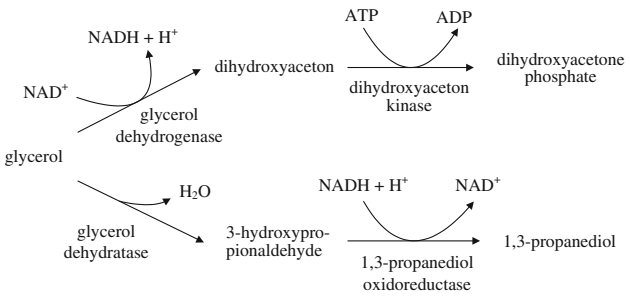

Fig. 1 1,3-Propanediol synthesis pathway

Materials and methods

Microorganism and growth conditions. *Kp* TUAC01 was kept at 4 °C storage. Preculture medium contained: $(NH_4)_2SO_4$·2 g/L, K_2HPO_4·$3H_2O$ 3.4 g/L, $KH_2PO_4$1.3 g/L, $MgSO_4$ 0.2 g/L, yeast extract 1 g/L, glycerol 30 g/L. Preculture was carried out in 250-mL flasks filled with 50 mL medium. Flasks were incubated in a rotary shaker at 37 °C and 120 rpm for 14 h. In fermentation culture, 50 mL of preculture was inoculated to a 5-L bioreactor (BIOSTAT-B B.Braun Germany) with the working volume of 4 L. The fermentation was kept steadily at pH 6.8 and 37 °C. Medium used here was: $(NH_4)_2SO_4$ 4 g/L, K_2HPO_4 ·$3H_2O$ 0.69 g/L, KH_2PO_4 0.25 g/L, $MgSO_4$ 0.2 g/L, yeast extract 1.5 g/L, glycerol 30 or 50 g/L, trace element solution 1 mL. One liter of trace element solution contained: $MnSO_4$·$4H_2O$ 100 mg, $ZnCl_2$ 70 mg, Na_2MoO_4·$2H_2O$ 35 mg, H_3BO_3 60 mg, $CoCl_2$·$6H_2O$ 200 mg, $CuSO_4$·$5H_2O$ 29.28 mg, $NiCl_2$·$6H_2O$ 25 mg, 37% HCl 0.9 mL. 40% (w/w) NaOH was used to adjust pH. Air feeding and rotation rate were 2 L/min and 250 rpm, respectively.

Quantitative determination

Biomass concentration was measured by optical density (OD) method at 640 nm. The broth component was measured by a Shimadzu 10AVP high performance liquid chromatograph system (Shimadzu Corp., Kyoto, Japan) with a RID-10A refractive index detector and an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, USA). The mobile phase was 0.005 mol/L H_2SO_4 solution, and the velocity was 0.8 mL/min. The column temperature was controlled at 65 °C.

Table 1 Different fermentation method for 1,3-propanediol production

Strain	Fermentation method	Final pdo (g/L)	Productivity (g/L per h)	Yield (mol/mol)	References
Citrobacter freundii	Continuous two-stage	41.5	1.38	0.62	[8]
Citrobacter freundii	Immobilized cell	16.4	8.2	0.57	[24]
Clostridium butyricum	Fed-batch	70.4	1.41	0.68	[25]
Clostridium butyricum	Continuous with cell recycling	20.9	20.9	0.61	[26]
Clostridium butyricum	Continuous two-stage	43.5	1.33	0.59	[21]
Klebsiella oxytoca	Fed-batch	83.5	1.61	0.62	[27]
Klebsiella pneumoniae	Fed-batch	73.3	0.92	0.48	[9]
Klebsiella pneumoniae	Fed-batch	75	2.2	0.61	[18]
Klebsiella pneumoniae	Micro-aerobic fed-batch	72	2.1	0.57	[18]
Klebsiella pneumoniae	Continuous	48.5	4.9	0.61	[19]
Klebsiella pneumoniae	Immobilized cell	4.1	16.4	0.30	[29]

3-HPA was measured by the tryptophane colorimetry method, described by Cirde [10].

Enzyme assays

The enzyme activities of glycerol dehydrogenase (EC 1.1.1.6), 1,3-propanediol oxidoreductase (EC 1.1.1.202) and glycerol dehydratase (EC 4.2.1.30) were assayed according to the methods described by Forage and Lin, Johnson and Ahrens, but with some modification [3, 12, 13]. The apparent activity of 1,3-propanediol oxidoreductase was determined using the reverse reaction, with a conversion factor of 3.95 for the physiological reaction. 1,2-Propanediol was used as substrate for the assay of glycerol dehydratase, and the conversion factor was 1.41. All the enzyme assays were carried out in a pH 7.0 phosphate buffer, which was mostly different from the literature methods. In the literatures, glycerol dehydrogenase and 1,3-propanediol oxidoreductase were assayed in a potassium carbonate buffer with pH 9.8 and 9.0, respectively. The enzyme activities presented in this paper were in the form of total enzyme activity in 1 mL fermentation broth (U/mL).

Nucleotide was extracted and assayed according to the method described by Abbad-Andaloussi et al. The nucleotide pools were presented in the form of the nucleotide amount per liter fermentation broth (μmol/L) [1].

Results and discussion

Effect of initial glycerol level on the accumulation of 3-HPA

Batch fermentations were carried out with 30 and 50 g/L of initial glycerol, results were shown in Fig. 2. In the batch of 50 g/L initial glycerol, the fermentation was ceased at 9 h, which was coincident with the peak of 3-HPA (12.6 mmol/L) in culture medium. While in the batch of 30 g/L initial glycerol, glycerol was completely consumed at 11 h. There was also a 3-HPA peak (7.5 mmol/L) at 7 h, but the level of 3-HPA in fermentation broth was gradually decreasing to 0 after 7 h. The yield of glycerol to 1,3-propanediol was not significantly different between the two batches, 0.62 and 0.63 (mol/mol), respectively.

The fermentation cessation in the batch of 50 g/L initial glycerol was caused by the toxic of 3-HPA, which indicated the peak concentration of 3-HPA (12.6 mmol/L) was a lethal dose to the cell. The 7.5 mmol/L of 3-HPA in the batch of 30 g/L initial glycerol did not affect cell growth, which indicated this level of 3-HPA was safe to cell. So the critical lethal level of 3-HPA to cells was in the range of 7.5–12.6 mmol/L, further experiment data obtained from our research indicated the critical lethal level was around 10 mmol/Ll. The level of 3-HPA decreased after the peak in the 30 g/L initial glycerol fermentation, implied the 3-HPA secreted in the broth could be re-assimilated by cells and further converted to 1,3-propanediol.

The amount of living cell in culture medium was measured before and after the cessation in the fermentation of 50 g/L initial glycerol. At 8 h, the amount of living cell was 1.5×10^9/mL, but at 10 h, the amount of survived cell was only 4.0×10^4/mL. Most of the cells were dead after the cessation, so the cessation was irreversible. The ceased fermentation broth was adjusted to glycerol concentration of 30 g/L, then reused as medium for new fermentation by sterilization and inoculation again. It was found that cell could grow in the reused medium, but less 1,3-propanediol (ac 5 g/L) was produced. So the accumulation of 3-HPA in broth was a great hazard to 1,3-propanediol production.

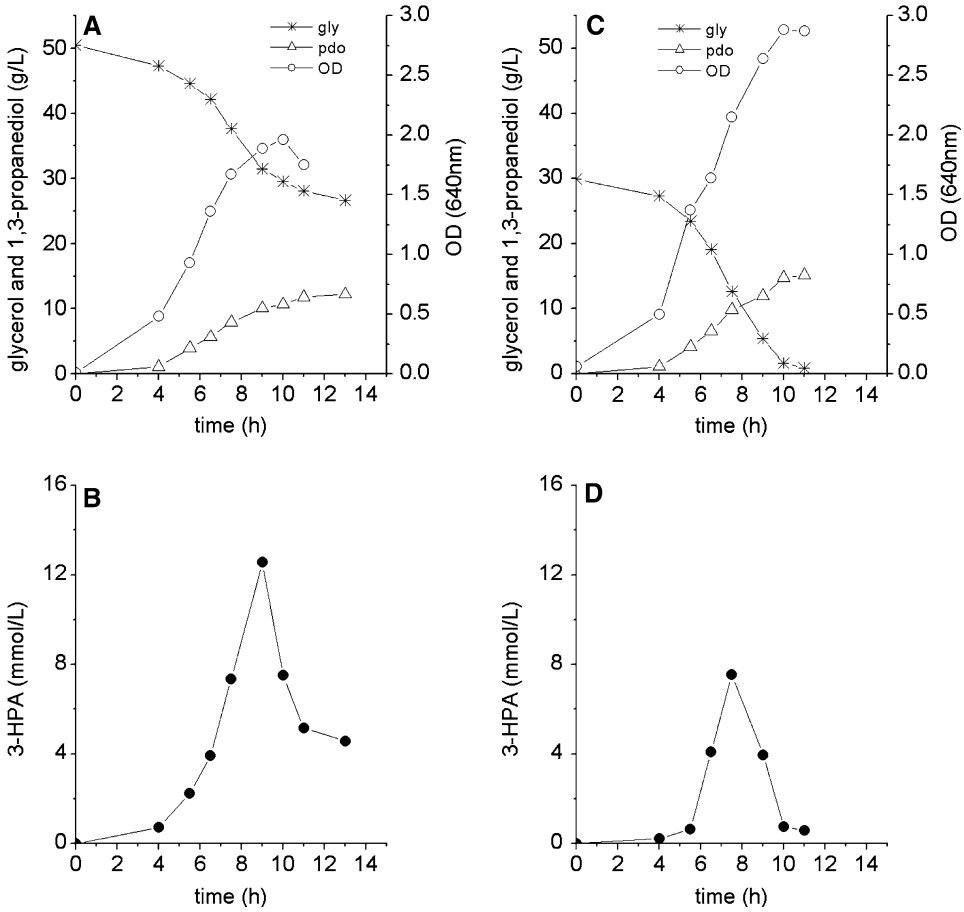

Fig. 2 The effect of the initial glycerol concentration on the 3-HPA accumulation. **a** Curves of glycerol consumption, 1,3-propanediol production and cell growth with initial glycerol 50 g/L. yield: 0.62 (mol/mol), productivity: 0.95 g/L per hour. **b** Curve of 3-HPA concentration with 50 g/L initial glycerol. **c** Curves of glycerol consumption, 1,3-propanediol production and cell growth with 30 g/L initial glycerol. yield: 0.63 (mol/mol), productivity: 1.37 g/L per hour. **d** Curve of 3-HPA concentration with 30 g/Ll initial glycerol. glycerol (*gly*), 1,3-propanediol (*pdo*), 3-HPA (HPA)

Effects of the 3-HPA on *dha* regulon enzymes

The activities of the three enzymes in *dha* regulon and the nucleotide pools in the two batch fermentations were measured, results were illustrated in Fig. 3. In the batch of 50 g/L initial glycerol, the activities of the three enzymes increased from 0 to 7.5 h, then the activities of 1,3-propanediol oxidoreductase and glycerol dehydratase decreased to a lower level, glycerol dehydrogenase activity decreased first but gradually resumed. The decreasing of enzymes activities coincided with the peak of 3-HPA in culture medium, which indicated the decreasing was caused by the toxicity of 3-HPA, and also indicated 1,3-propanediol oxidoreductase and glycerol dehydratase appeared more sensitive to the toxicity. In the batch of 30 g/L initial glycerol, the activities of the three enzymes were increasing continuously in the first 10 h, but they all decreased to a lower level later. Though there was a 3-HPA peak at 7.5 h too, the activities decrease just followed the exhaustion of glycerol, it seems the activities of the three enzymes were not affected at the level of 7.5 mmol/L.

The level of NAD^+ and NADH was increasing with the cell growth, and there appeared few different between the two batches. The level of NAD^+ was higher than NADH, and the ratio of NADH to NAD^+ was stable around 0.5–0.8 in the two batches, the intracellular concentration of NAD^+ and NADH was 1.12 and 0.64 μmol/g DW, respectively. Both the intracellular nucleotide concentration and the ratio of NADH to NAD^+ were lower than *K. pneumoniae* DSMZ and *C. butyricum* DSM 541 in literatures. Menzel [20] reported the intracellular concentration of NAD^+ and NADH was 5 and 4 μmol/g DW in the continuous culture of *K. pneumoniae* DSMZ 2026, the ratio of NADH to NAD^+ was around 0.8–0.95. Abbad-Andaloussi [1] reported the intracellular concentration of NAD^+ and NADH was 9 and 20 μmol/g DW in continuous culture of *C. butyricum* DSM 541, the ratio of NADH to NAD^+ was 2.1–2.3. The higher ratio was one of the reasons they stated glycerol dehydratase was the rate limiting step in 1,3-propanediol producing, but it seems different for *KP*TUAC01. In the investigation of utilize glucose as subtract to produce 1,3-propanediol, a non-special alcohol dehydrogenase was used to replace the 1,3-propanediol oxidoreductase in reconstructed *E. coli*. This non-special alcohol dehydrogenase utilize NADPH other than NADH in convert 3-HPA to 1,3-propanediol, for the ratio of NADPH to $NADP^+$ is higher than the ratio of NADH to NAD^+ in cell, a very higher level of 1,3-propanedil (130 g/L) was achieved by

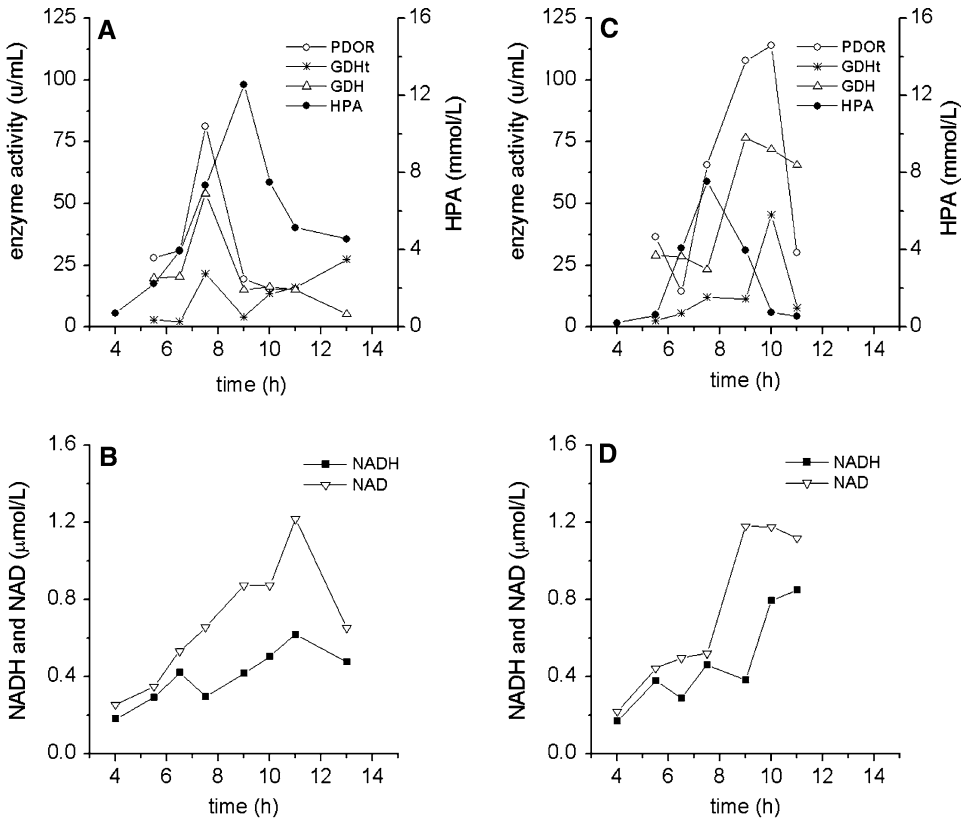

Fig. 3 The effect of 3-HPA on the three key enzymes of *dha* regulon. **a** Enzymes activity curves and 3-HPA curve in the fermentation with 50 g/L initial glycerol. **b** NAD and NADH curves in the fermentation with 50 g/L initial glycerol. **c** Enzymes activity curves and 3-HPA curve in the fermentation with 30 g/L initial glycerol. **d** NAD and NADH curves in the fermentation with 30 g/L initial glycerol. 3-HPA (*HPA*), glycerol dehydrogenase (*GDH*), 1,3-propanediol oxidoreductase (*PDOR*), glycerol dehydratase (*GDHt*)

this constructed strain [21]. It implicated the ratio of NADH to NAD^+ was a limited factor in the 1,3-propanediol production in *E. coli*.

The activity of glycerol dehydratase was slight higher than 1,3-propanediol oxidoreductase, which was different to the results of Ahrens et al. In their reports, the activity of glycerol dehydratase was ten times lower than the activity of 1,3-propanediol oxidoreductase, it was deduced that the glycerol dehydratase was the major rate limiting enzyme for the glycerol dissimilation [1, 3, 8]. There was different result about the activities of glycerol dehydratase and 1,3-propanediol oxidoreductase, which came from our modified enzyme assay method. pH 7.0 buffer rather than pH 9.0 or 9.8 buffer was used for 1,3-propanediol oxidoreductase assay in our experiment. In the higher pH like 9.0 or 9.8, the enzyme assay showed higher activity, but we considered the cytoplasm pH might be more near to 7.0 than 9.0, so pH 7.0 buffer was selected. Our result indicated the activity of glycerol dehydratase and 1,3-propanediol oxidoreductase was in the same order of magnitude, it was difficult to deduce which enzyme was the rate limiting enzyme just from the result of enzyme activity assays. Considered the aspect of nucleotide pools, the 1,3-propanediol oxidoreductase might prospectively be the rate limiting enzyme. Anyway, the 3-HPA accumulation in fermentation broth indicated: the reaction catalyzed by 1,3-propanediol oxidoreductase was the rate limiting step in the cell exponential grow phase.

The activities revival of the three *dha* regulon enzymes

To get further information about the effect of 3-HPA on the three enzymes, fermentation with pulsive glycerol feeding was carried out, see Fig. 4. Large amount of glycerol was fed to the culture medium in a minute, which brought a pulse of glycerol level in culture medium. Two glycerol pulses were created in the fermentation process, the first one was at 11.5 h and the second was at 12.5 h. In the phase of 7.5–11.5 h, residual glycerol in broth was lower than 10 g/L and kept on consuming, the 3-HPA level in broth was decreasing accordingly. The activity of the three enzymes increased with cell growth and glycerol consumption, accompanying with glycerol was exhausted completely, the enzyme activities was gradually decreasing to a lower level. Corresponding to the first glycerol pulse at 11.5 h, the activity of the three enzymes returned to the original level before glycerol was exhausted. The prompt revival of the enzyme activities indicated that they were controlled on metabolic levels, but not on the genetic levels which related to enzyme syntheses. The decrease of enzyme activities caused by glycerol exhaustion was reversible. After the second glycerol pulse, the activities of 1,3-propanediol oxidoreductase and glycerol dehydratase could not return to a higher level but decreased to a very lower level. At the same time, 3-HPA increased to a higher level (11.5 mmol/L) in a very short time (<5 min after the second glycerol pulse). Both 1,3-propanediol

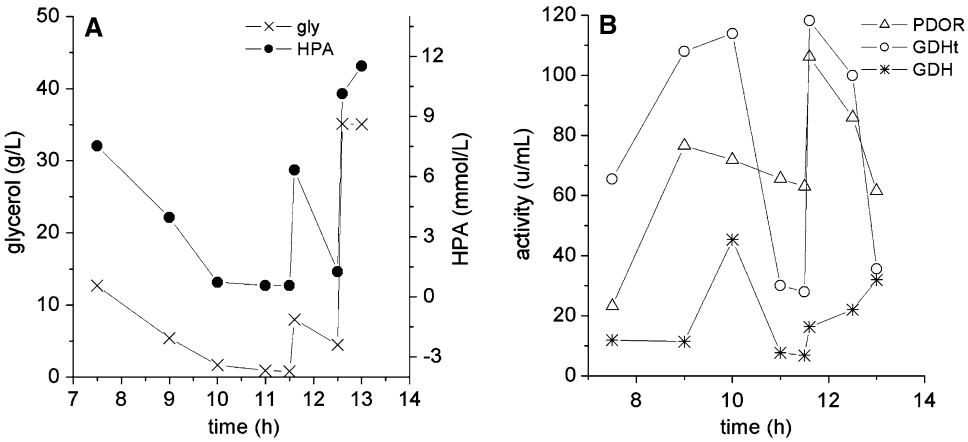

Fig. 4 Activities revival of the *dha* regulon key enzymes in fermentation. Glycerol pulse was created at 11.5 and 12.5 h, the activities lost caused by the exhaustion of substrate was reversible, the activities lost caused by the toxicity of 3-HPA was irreversible for 1,3-propanediol oxidoreductase and glycerol dehydratase. glycerol (*gly*), 3-HPA (*hpa*), glycerol dehydrogenase (*GDH*), 1,3-propanediol oxidoreductase (*PDOR*), glycerol dehydratase (*GDHt*)

oxidoreductase and glycerol dehydratase were sensitive to high concentration of 3-HPA. When the level of 3-HPA in broth was higher than 10 mmol/L, the activities of the two enzymes were lost irreversibly. This kind of enzyme activity lost was different to the enzyme activity decrease caused by the exhaustion of glycerol, which was reversible.

Ahrens stated that the reductive pathway of glycerol dissimilation was largely controlled by the synthesis of enzymes on genetic levels. Their results came from the investigation on continuous culture of *K. pneumoniae* DSM 2026 [3]. The productivity of 1,3-propanediol in continuous culture was higher than in batch culture, so enzyme activities in vivo (based on metabolic flux analysis) was higher. Then the ratio of enzyme activities in vitro (based on enzyme assay) to enzyme activities in vivo was lower and near 1. In our experiments of batch culture, the in vivo enzyme activities of 1,3-propanediol oxidoreductase and glycerol dehydratase, calculated from 1,3-propanediol productivity, were about 0.5 U/mL, which are 30–200 times lower than the activities in vitro. Combined with the phenomenon of enzyme activities quickly revived after glycerol supplement, it could be concluded that enzyme syntheses did not play a key role on the enzyme activities regulation in the fed bath fermentation of *Kp*TUAC01 The enzyme activities regulation was mainly adjusted on metabolic levels.

As the 3-HPA level in culture medium was controlled by the rate of its production and consumption, and glycerol played a key role on the regulation of the activities of 1,3-propanediol oxidoreductase and glycerol dehydratase, which catalyzed 3-HPA production and consumption, respectively, so glycerol could be used as an adjustable factor to control the 3-HPA level in broth.

Feeding glycerol to control the 3-HPA level in fed batch fermentation

The effect of glycerol concentration on 3-HPA accumulation in fed batch fermentations was studied with five feeding modes, and results were shown in Fig. 5. In the first mode, glycerol feeding started at 8 h, at that time the residual glycerol in broth was 8.9 g/L. After that, the glycerol level was kept around 8 g/L by adjusting the feeding rate. The level of 3-HPA in broth was increasing with glycerol consumption, after reaching a peak at 11 h (8.6 mmol/L), it began to decreased. In the modes of second and third the glycerol feeding began at 9 and 11 h separately, and the residual glycerol in culture medium was 2.4 and 1.5 g/L, respectively. After feeding took place, the glycerol level was maintained around 7 and 3 g/L, respectively. In the two modes, the level of 3-HPA in culture medium had already run across a peak before glycerol feeding started. Due to glycerol feeding, another 3-HPA peak appeared at 12 and 14 h, to a level of 4.6 and 1.9 mmol/L, respectively.

Glycerol feeding began at 10 h in both the modes of fourth and fifth. The residual glycerol in culture medium was separately 1.5 and 1.9 g/L at that time, then the glycerol concentration in both modes was increasing gradually depended on the feeding rate. In the fourth case, 3-HPA in broth was gradually increasing after feed beginning and reached the second peak at 18 h, then the fermentation was ceased due to the toxicity of high concentration 3-HPA. In the fifth case, glycerol level in broth was lower than the fourth case. Though the glycerol level in culture medium was higher than 10 g/L from 15 to 20 h, 3-HPA level was maintained at a low level till the end of fermentation.

The above results indicated the level of 3-HPA in culture medium could be controlled by adjusting glycerol feeding strategy. In the cell exponential grown phase (ac from 7 to 20 h) of fed batch fermentation, higher concentration of glycerol in medium resulted in higher concentration of 3-HPA accumulation. Starting glycerol feed at a concentration of residual glycerol lower than 7–8 g/L, and keeping this lower glycerol level further to 20 h, fermentation would not accumulate 3-HPA to a lethal level.

On the other hand, 3-HPA was the direct substrate for 1,3-propanediol production, lower 3-HPA level would

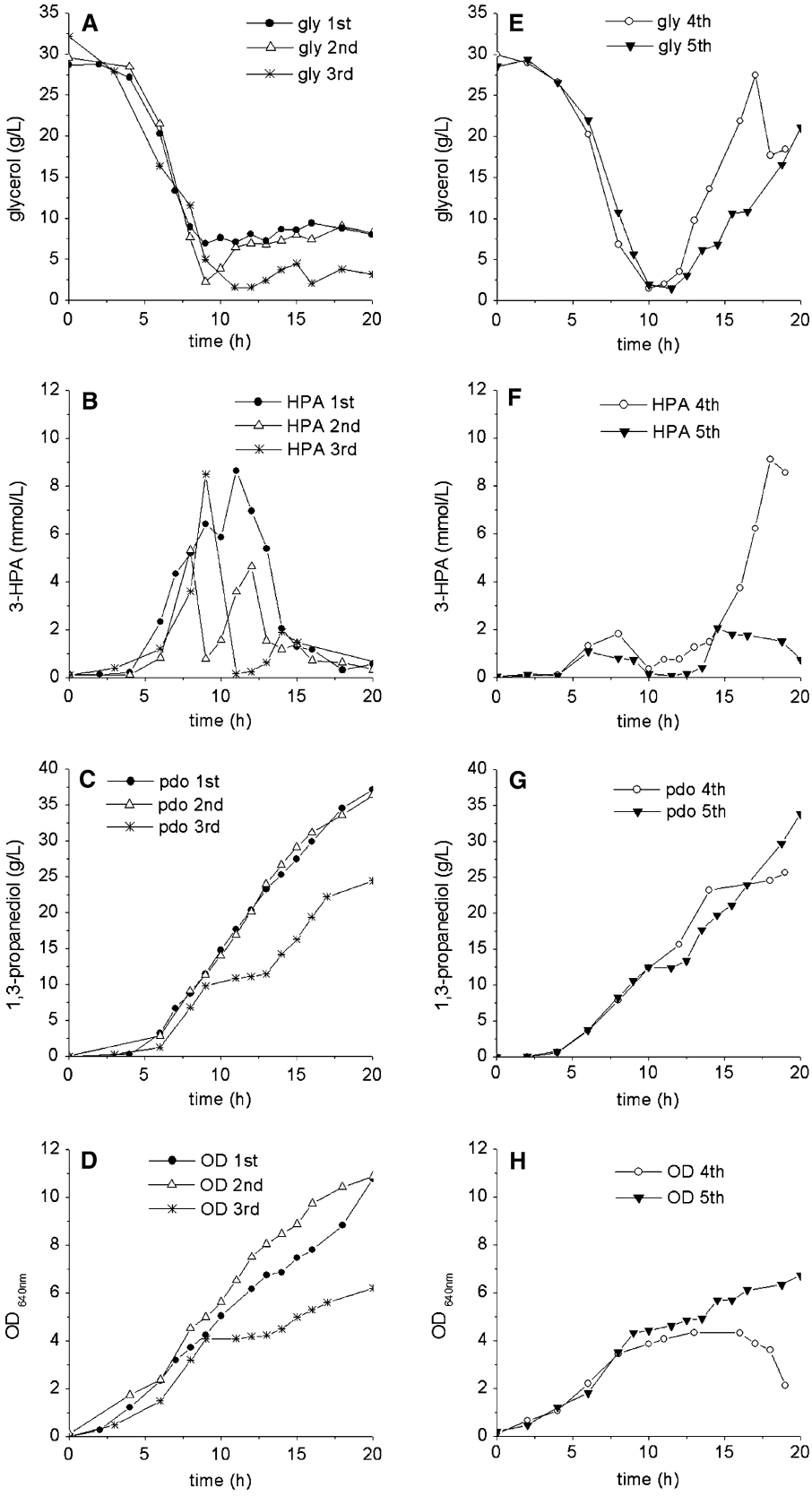

Fig. 5 The effect of glycerol concentration on 3-HPA accumulation in fed batch fermentation. **a**–**d** The glycerol, 3-HPA, 1,3-propanediol and OD curves in modes first, second and third fermentations. **e**–**h** The glycerol, 3-HPA, 1,3-propanediol and OD curves in modes fourth and fifth fermentation. In the first, second and third cases, glycerol feeding was started at 8, 9 and 11 h, respectively, glycerol concentration was kept stable at 8, 7 and 3 g/L, respectively. In modes fourth and fifth fermentation, glycerol feeding was started at 10 h, and glycerol concentration increased gradually

prevent higher 1,3-propanediol productivity to be achieved, which could be given clear from the third and the fourth fermentation. The level of glycerol in both cases was lower than 2 g/L at 10 h, and the level of 3-HPA was only 0.16 and 0.36 mmol/L, respectively, there appeared a halt on 1,3-propanediol production and cell growth at that time in the two fermentations. Lower level of glycerol made the step of conversion glycerol into 3-HPA become the rate limiting step for 1,3-propanediol production, low glycerol concentration also affected cell growth. So that, under the precondition of avoiding accumulation 3-HPA to a lethal level, a high-producing fed batch fermentation should keep glycerol at a relative higher level.

Fed batch fermentation in optimum conditions

Based on the experiments mentioned above, a whole fed batch fermentation process was carried out, result was shown in Fig. 6. Glycerol feeding was started at 10 h. 3-HPA reached its second peak at 16 h, and the level of glycerol was 9 g/L at that time. The level of glycerol in culture medium was continuously increasing, and reached the highest concentration (43.7 g/L) at 40 h. 1,3-propanediol concentration in broth was quickly increasing from 6 to 24 h, the cell exponential grow phase of the whole fermentation. After that, 1,3-propanediol increased at a lower rate, but by-products shared a higher producing rate. The 1,3-propanediol concentration reached to 51.1 g/L at 24 h, and its final concentration was 73.1 g/L at 54 h.

The fermentation was repeated times, the formation trend of 1,3-propanediol and the by-products was similar to the curves in Fig. 6. The results of eight fermentation processes were shown in Table 2. 1,3-propanediol was produced mainly in early 36 h, after that, the 1,3-propanediol concentration was still increasing, but not too much. In addition, the yield decreased with fermentation time being prolonged, for the reason of more by-products being produced. According to the investigation, the fermentation has been successfully scaled up to an industrial level of 50 m^3 by our research team cooperated with a company.

Conclusion

Unlike other reported microorganisms that produced 1,3-propanediol strict anaerobically, *Kp*TUAC01 was an aerobic

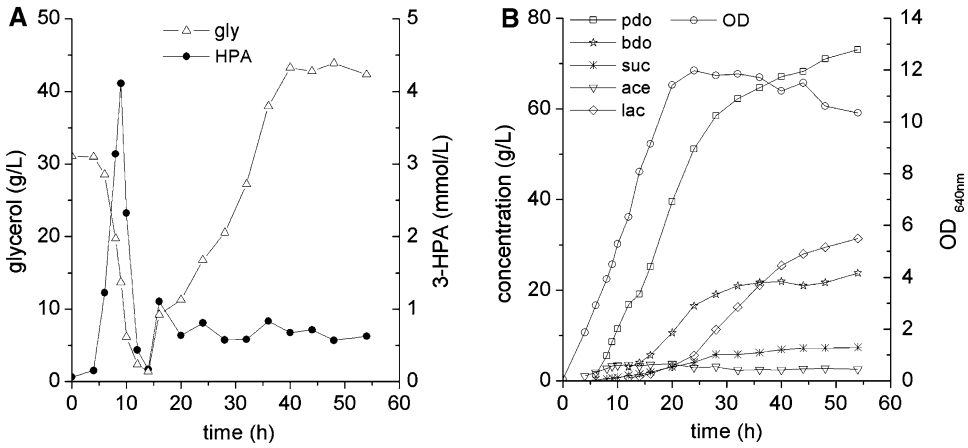

Fig. 6 Optimized aerobic fed batch fermentation process of TUAC01. **a** Glycerol and 3-HPA curves in the process. **b** OD, 1,3-propanediol and by products curves in broth. Glycerol (*gly*), 1,3-propanediol (*pdo*), lactic acid (*lac*), succinic acid (*suc*), acetic acid (*ace*), 2,3-butanediol (*bdo*)

Table 2 The yields and productivities of the fermentations in optimized condition

Batch	36 h			48 h			Final 60–72 h		
	Pdo (g/L)	Productivity (g/L/h)	Yield (mol/mol)	Pdo (g/L)	Productivity (g/L/h)	Yield (mol/mol)	Pdo (g/L)	Productivity (g/L/h)	Yield (mol/mol)
1	59.9	1.67	0.56	60.1	1.25	0.53	64.5	1.08	0.53
2	57.9	1.61	0.59	61.8	1.29	0.59	64.9	1.08	0.56
3	58.6	1.63	0.52	64.2	1.34	0.50	66.9	0.93	0.49
4	61.2	1.70	0.59	63.8	1.33	0.57	67.8	1.00	0.56
5	62.1	1.72	0.59	68.5	1.43	0.59	71.4	0.99	0.56
6	64.5	1.79	0.63	70.2	1.46	0.60	71.7	1.05	0.61
7	64.7	1.80	0.60	71.2	1.48	0.60	75.6	1.05	0.59
8	73.6	2.05	0.61	74.8	1.56	0.56	81.9	1.20	0.56
Average	62.8	1.75	0.59	66.8	1.39	0.57	70.6	1.05	0.56

1,3-propanediol producing strain. The toxicity of 3-HPA in anaerobic microorganism conversion of glycerol to 1,3-propanediol was reported. The results got in this paper indicated the toxicity was also existed in aerobic 1,3-propanediol production. 3-HPA level was controlled by the rate of its production and consumption. 3-HPA accumulation in 1,3-propanediol fermentation was by a positive feedback mechanism, the toxicity of 3-HPA led to activity lost of the 1,3-propanediol oxidoreductase, which furthermore hindered the reaction of converting 3-HPA to 1,3-propanediol. The limiting step for 1,3-propanediol production by *Kp*TUAC01 in aerobic fed batch fermentation might be different in different cell growth phase, the step of conversion 3-HPA to 1,3-propanediol was the limiting step in the exponential growth phase. Glycerol was the substrate of 3-HPA and played key role in regulating the activities of 1,3-propanediol oxidoreductase and glycerol dehydratase. So adjusting the glycerol level in medium could be used to control the 3-HPA level. In fed batch fermentation, the safe initial glycerol concentration was around 30 g/L, after the glycerol had been consumed lower than 7–8 g/L, the glycerol feeding could be started. In the whole phase of exponential growth, the glycerol level should be kept lower than 7–8 g/L, under this condition, a safe process with higher 1,3-propanediol productivity was set up.

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