J Ind Microbiol Biotechnol (2008) 35:735–741
DOI 10.1007/s10295-008-0340-y

ORIGINAL PAPER

Decrease of 3-hydroxypropionaldehyde accumulation in 1,3-propanediol production by over-expressing *dha*T gene in *Klebsiella pneumoniae* TUAC01

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Received: 25 June 2007 / Accepted: 3 March 2008 / Published online: 26 March 2008

Abstract Glycerol can be biologically converted to 1,3-propanediol, a key raw material required for the synthesis of polytrimethylene terephthalate and other polyester fibers. In 1,3-propanediol synthesis pathway, 3-hydroxypropionaldehyde (3-HPA) was an inhibitory intermediary metabolite. The accumulation of 3-HPA in broth would cause an irreversible cessation of the fermentation process. With the object of reducing 3-HPA level in the fermentation broth, *dha*T gene which encodes 1,3-propanediol oxidoreductase (PDOR) was cloned and over expressed in 1,3-propanediol producing bacterium *Klebsiella pneumoniae* TUAC01. *dha*T gene was linked downstream of the ptac promoter in an expressing vector pDK6 to form plasmid pDK-*dha*T. The newly formed pDK-*dha*T was transformed to *K. pneumoniae* TUAC01. Under the inducement of IPTG, PDOR was over-expressed when the constructed strain was cultured on an LB medium or a fermentation medium. A 5 L scale-up fermentation experiment was done to test the 3-HPA accumulation in broth, with the initial substrate glycerol 30 g/L; the peak levels of 3-HPA in broth were 7.55 and 1.49 mmol/L for control host strain and the constructed strain, respectively. In 50 g/L initial glycerol experiment, the peak level of 3-HPA in broth was 12.57 and 2.02 mmol/l for the control host strain and the constructed strain, respectively. Thus the fermentation cessation caused by the toxicity of 3-HPA was alleviated in the constructed strain.

Keywords *dha*T · 1,3-Propanediol · 3-Hydroxypropionaldehyde · *Klebsiella pneumoniae*

Abbreviations

Ace	Acetate acid
Bdo	Butanediol
Eth	Ethanol
Gly	Glycerol
3-HPA	3-Hydroxypropionaldehyde
Lac	Lactate acid
Pdo	1,3-Propanediol
Suc	Succinic acid
PDOR	1,3-Propanediol oxidoreductase

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Introduction

1,3-Propanediol, a bulk chemical, can be formulated into a variety of industrial products especially in the synthesis of polytrimethylene terephthalate and other polyester fibers [18]. 1,3-Propanediol can be produced either by chemical synthesis or by microbial fermentation. Current commercial routes to produce 1,3-propanediol are chemical synthetic methods using acrolein or ethylene oxide as substrates [2, 13]. In resent years the advantage of utilizing inexpensive, renewable resource such as glycerol as the substrate,

biological route to produce 1,3-propanediol has become increasingly attractive.

3-Hydroxypropionaldehyde (3-HPA) is an intermediary metabolite in 1,3-propanediol synthesis pathway. It is also an inhibitor to bacteria; high level of 3-HPA in culture broth would cause the cessation of 1,3-propanediol production and microorganism growth [3]. In the metabolic pathway of glycerol conversion to 1,3-propanediol, glycerol is first converted to 3-HPA under the catalysis of glycerol dehydratase, then 3-HPA dehydrogenated to 1,3-propanediol which is catalyzed by 1,3-propanediol oxidoreductase (PDOR) (Fig. 1) [10]. The accumulation of 3-HPA in broth is caused by a faster formation rate when companied with a lower consumption rate, and it is related to the ratio of glycerol dehydratase activity to PDOR activity. On the other hand, 3-HPA has an inhibitory effect on glycerol dehydratase, the low rate of glycerol dissimilation following the appearance of 3-HPA in the culture broth is attributed to this strong inhibition [4]. So there appears a negative feedback in the 3-HPA accumulation. In our previous investigation, it was found that the PDOR was more sensitive to higher levels of 3-HPA. Thus if 3-HPA increased to a certain higher level, then it cannot be converted to downstream product 1,3-propanediol for the lost activity of PDOR.

The exact antimicrobial mechanism of 3-HPA is still not clear. One hypothesis is the three-dimensional molecular model of 3-HPA dimer that closely resembled D-ribose. It was then postulated that 3-HPA dimer is able to compete with ribonucleotides on binding to the ribose recognition site of ribonucleotide reductase and inhibit the conversion of ribonucleotides into deoxyribonucleotides. Another hypothesis involves the functional groups of the 3-HPA. The hydroxyl and the aldehyde groups might be responsible for the relative reactivity of the 3-HPA, which might cause it to react with unstable sulfhydryl groups of ribonucleotide reductase and thioredoxin [17].

We have isolated an aerobic 1,3-propanediol-producing strain, which belonged to the species of *Klebsiella pneumoniae subspecies Pneumoniae* and was named *Kp*TUAC01 [11]. In our preliminary investigation about the aerobic 1,3-propanediol produced by *Kp*TUAC01, it was found the 3-HPA can be accumulated in the fermentation broth; sometimes 3-HPA accumulated in a high level and ceased the fermentation. This cessation in fermentation is irreversible, so this cessation should be completely avoided in 1,3-propanediol production. We have found that the accumulation of 3-HPA in broth was related to the glycerol level, and the pH of medium [14]. In this paper we try using a genetic method to reduce 3-HPA accumulation, for the 3-HPA level in broth was controlled by the formation rate and the consumption rate; if the consumption rate is enhanced then the accumulation of the 3-HPA would be alleviated. With this goal the *dhaT* gene, which encodes the PDOR, is cloned and over-expressed in the 1,3-propanediol-producing strain *Kp*TUAC01.

Materials and methods

Microorganism and plasmids

Klebsiella pneumoniae subspecies Pneumoniae TUAC01 (*Kp*TUAC01) and other transformed strains were stocked at 4 °C. Plasmids used in this paper are listed in Table 1.

Construction of expressing vectors

Standard techniques of recombinant DNA technology were used for DNA manipulations [15]. Restriction and DNA-modifying enzymes were obtained from TaKaRa (Dalian, China) and used according to the instructions of the manufacturer.

*Kp*TUAC01 was a strain resistanct to ampicillin, so a kanamycin resistance vector pDK6 was used as an expression vector. The *dhaT* gene was amplified from genomic DNA of *Kp*TUAC01 by PCR, using forward (5′-CGAATT CCGCATTATAACCTGAAGCG-3′) and reverse (5′-TAA AGCTTCTCAGAATGCCTGGCGGA-3′) primers with EcoRI and HindIII restriction sites, respectively. Primers

Table 1 Cloning vectors used and plasmids cited in the text

Plasmid	Properties	Source or reference
pMD18-T-Simple	Amp^r, 2692pb *E. coli* cloning vector	TaKaRa
pMD-dhaT	Plasmid pMD18-T-Simple containing *dhaT* gene	This work
pDK6	Expressing vector, Km^r, 5.1 kb	Kleiner et al. [12]
pDK-dhaT	Plasmid pDK6 containing *dhaT* gene	This work

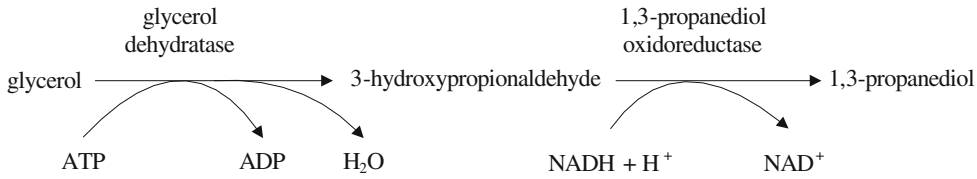

Fig. 1 The metabolic pathway of glycerol convertion to 1,3-propanediol

were designed according to the *dhaT* gene of *K. pneumoniae* DSM 2026 (P. Zheng et al. submitted in Gene Bank). This PCR product contains the whole ORF of *dhaT* gene and 45 upstream nucleotides, after the agarose gel-purified fragment was cloned to pMD18-T-Simple vector to create pMD-dhaT and determinate the *dhaT* gene sequence. Amplified pMD-dhaT was digested with EcoRI/HindIII, the *dhaT* gene containing fragment was agarose gel-purified and inserted into the EcoRI and HindIII sites of the pDK6 expression vector to create pDK-dhaT. The *dhaT* gene in pDK-dhaT was under the control of a Ptac promoter. The pDK-dhaT construct plasmid was first transformed into *Escherichia coli* dH5α for amplifing, using the freeze/thaw method. The resultant strain, *E. coli*/pDK-*dha*T, was kanamycin resistant. Then the amplified plasmid pDK-*dha*T was electro-transformed back into *Kp*TUAC01 to create the resultant strain *Kp*/pDK-*dha*T.

Nucleotide sequence accession number. The cloned *dhaT* gene nucleotide sequence was submitted to GenBank under the accession No. EF424558.

dhaT Gene expression experiment

The resultant strains *E. coli*/pDK-dhaT and *Kp*/pDK-dhaT were cultured in a rotary shaker at 37 °C and 120 rpm, using LB medium; after 10 h culture, 1 mmol/l of isopropyl-*β*-D-thiogalactoside (IPTG) was added to the medium to induce the gene expression; the activity of PDOR in cell extract was detected 5 h later.

Enzymes assay

The enzyme assay of PDOR (EC 1.1.1.202) was done according to the method described by Forage and Lin [10] and Ahrens [1].

Fermentation experiment

Flask fermentation experiment using 250-mL flasks filled with 50 mL medium, incubated in a rotary shaker at 37 °C and 120 rpm for 24 h, and with the addition of $CaCO_3$ was used to control the pH. Fermentation medium contained: $(NH_4)_2SO_4$ 4 g/l, $K_2HPO_4 \cdot 3H_2O$ 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: $MnSO_4 \cdot 4H_2O$ 100 mg, $ZnCl_2$ 70 mg, $Na_2MoO_4 \cdot 2H_2O$ 35 mg, H_3BO_3 60 mg, $CoCl_2 \cdot 6H_2O$ 200 mg, $CuSO_4 \cdot 5H_2O$ 29.28 mg, $NiCl_2 \cdot 6H_2O$ 25 mg, 37% HCl 0.9 mL.

For batch fermentation, a 5 L bioreactor (BIOSTAT-B B.Braun, Germany) was used, the working volume was 4 L. In the whole process the ferment broth was kept steadily at pH 6.8 and 37 °C. 40% (w/w) NaOH was used to adjust pH. Air feeding and rotation rate were 2 L/min and 250 rpm, respectively.

Ferment broth component analytical methods

The biomass concentration was measured as optical density (OD) 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. The stationary and mobile phases were an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, USA) and 0.005 mol/L H_2SO_4 solution at 0.8 mL/min, respectively. The column temperature was controlled at 65 °C.

3-HPA was measured according to the method described by Cirde et al. [8].

Results

Characterization of the *dhaT* gene

The *dhaT* gene amplified from *Kp*TUAC01 was sequenced. The ORF of *dhaT* gene was 1164 pb and the predicted amino acid results showed 387 amino acid residues. The *dhaT* gene and the coded protein were compared using blast in NCBI; the identities of the existing genes and proteins are listed in Table 2. The highest homologous gene was *dhaT* gene from *K. pneumoniae* DSM 2026, the primers used in amplifing *dhaT* gene was designed according to this sequence, was followed by the *dhaT* gene from two *Citrobacter freundii*. For the coded protein, *K. pneumoniae* ATCC 25955 has the highest homologous 1,3-propanediol dehydrogenase (another name of PDOR) to that of *Kp*TUAC01; PDOR from *K. pneumoniae* DSM 2026, *C. freundii*, and *Clostridium pasteurianum* also has a higher homology compared to the PDOR from *Kp*TUAC01.

dhaT gene expression in *E. coli* and *Kp*TUAC01

Constructed strains *E. coli*/pDK-dhaT and *Kp*/pDK-dhaT were cultured in the LB medium to test the *dhaT* gene expression. Non-induced fermentation and host strains were tested as control (Fig. 2). No PDOR activities were either tested in host cell *E. coli* dH5α or *Kp*TUAC01. A relatively low PDOR activity appeared in the non-induced resultant of both *E. coli*/pDK-dhaT and *Kp*/pDK-dhaT (0.48 and 0.09 U/mgP, respectively), whereas for those induced with IPTG a higher level of PDOR activity was measured in both *E. coli*/pDK-dhaT and *Kp*/pDK-dhaT (1.56 and 1.39 U/mgP, respectively).

Table 2 The blast results of *dhaT* gene sequence and amino acid sequence in NCBI

Definition	Genbank accession number	Identities	Strains	References
dhaT gene	AY340973, AJ567471	94% (1,105/1,165)	*K. pneumoniae* DSM 2026	Zheng et al. [19]
dhaT gene	DQ416747	94% (1,102/1,168)	*C. freundii*	
dhaT gene	U09771	86% (994/1,174)	*C. freundii* DSM 30040	Daniel et al. [9]
1,3-Propanediol dehydrogenase	Q59477	98% (382/387)	*K. pneumoniae* ATCC 25955	Skraly et al. [16]
1,3-Propanediol oxidoreductase	CAD98878	98% (380/387)	*K. pneumoniae* DSM 2026	
1,3-Propanediol dehydrogenase	ABD74004	94% (368/388)	*C. freundii*	
1,3-Propanediol oxidoreductase	AAS17877	93% (363/387)	*C. pasteurianum*	Chi et al. [7]

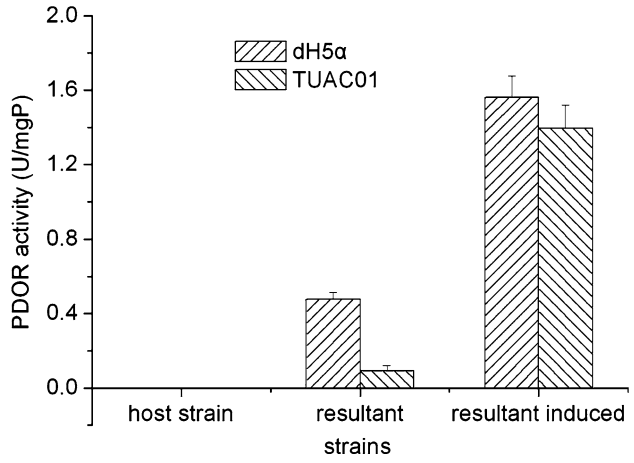

Fig. 2 *dhaT* gene expression in *E. coli* dH5α and *Kp*TUAC01. Both host strains *E. coli* dH5α and *Kp*TUAC01 have no PDOR activity cultured in LB medium. In non-induced condition resultant strains *E. coli*/pDK-dhaT and *Kp*/pDK-dhaT expressed the PDOR activity of 0.48 and 0.09 U/mgP, respectively. Induced by IPTG, *E. coli*/pDK-dhaT and *Kp*/pDK-dhaT expressed PDOR activity at 1.56 and 1.39 U/mgP, respectively. U/mgP means unit enzyme per 1 mg cell protein

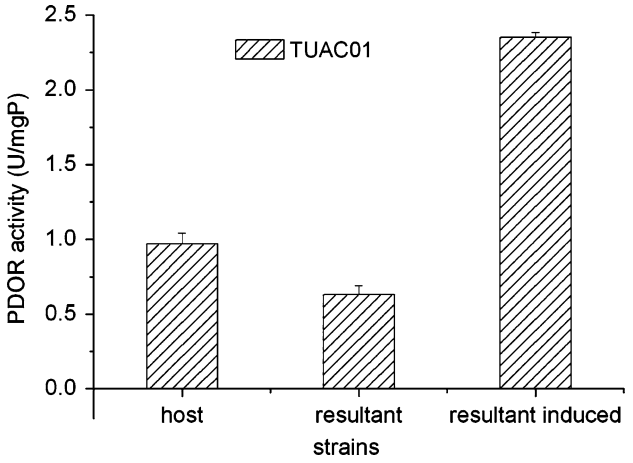

Fig. 3 PDOR activity of the resultant strain *Kp*/pDK-dhaT in flask fermentation. PDOR was measured at fermentation 12 h, *Kp*TUAC01 0.97 U/mgP; *Kp*/pDK-dhaT non-induced 0.63 U/mgP; *Kp*/pDK-dhaT IPTG induced 2.35 U/mgP

Flask fermentation of *Kp*/pDK-dhaT

Kp/pDK-dhaT and host *Kp*TUAC01 were cultured in flasks with fermentation medium. For *Kp*/pDK-dhaT, after 7 h culture 1 mg/mL IPTG was added to the culture medium to induce *dhaT* gene expression; host *Kp*TUAC01 and non-induced *Kp*/pDK-dhaT were tested as control. The activity of PDOR was detected at 12 h, and the final fermentation products were analyzed at 24 h. Results are shown in Figs. 3 and 4. *Kp*TUAC01 and *Kp*/pDK-dhaT show PDOR activity in 12 h culture, the IPTG-induced *Kp*/pDK-dhaT shows a PDOR activity twice higher than the host strain *Kp*TUAC01, whereas the non-induced *Kp*/pDK-dhaT exhibit a lower PDOR activity than the host strain *Kp*TUAC01.

The 1,3-propanediol produced had no distinct difference within the *Kp*TUAC01, *Kp*/pDK-dhaT, and IPTG-induced *Kp*/pDK-dhaT. The by-products showed some differences in the IPTG-induced *Kp*/pDK-dhaT. 2,3-Butanediol and succinate acid levels were higher in *Kp*/pDK-dhaT than in

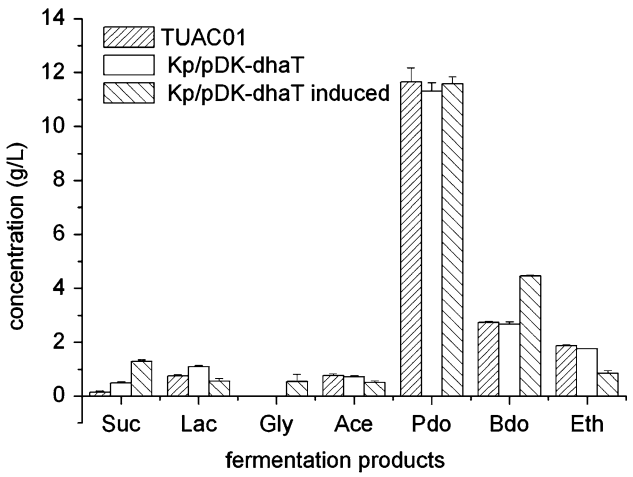

Fig. 4 Flask fermentation results of resultant strain *Kp*/pDK-dhaT and host strain *Kp*TUAC01. 1,3-propanediol shown distinct differences in the hosts *Kp*TUAC01 and *Kp*/pDK-dhaT; the by-products, succinate acid, and 2,3-butanediol levels were increased in the *Kp*/pDK-dhaT; all other by-products have a lower level than that of *Kp*TUAC01

*Kp*TUAC01; whereas lactic acid, acetic acid, and ethanol levels were lower than in *Kp*TUAC01. On the whole, the carbon atom in the substrate glycerol had a higher conver-

sion ratio when compared to the measured product and by-products in the induced *Kp*/pDK-dhaT (80%) than that in the non-induced condition (73%) and the host strain *Kp*TUAC01 (73%); the residual carbon atom from glycerol escaped with carbon dioxide. We should pay more attention to some glycerol that remained in the induced *Kp*/pDK-dhaT ferment broth.

Batch bioreactor fermentation

The main object of this work was to reduce the 3-HPA accumulation in broth, but in flask fermentations the 3-HPA level in broth was very low and had no distinction between strains. The batch fermentation in the bioreactor was used to detect the 3-HPA accumulation in *Kp*/pDK-dhaT. Initial glycerol experiments of 30 and 50 g/L were done; results are shown in Figs. 5 and 6. In *Kp*/pDK-dhaT fermentations 1 mmol/l IPTG was added to the broth at 4 h to induce the plasmid *dhaT* gene expression.

In the batch fermentation with 30 g/L initial glycerol in the medium, for both the strains *Kp*TUAC01 and *Kp*/pDK-dhaT, glycerol was gradually consumed and 1,3-propanediol was produced accordingly. The glycerol was exhausted at 11 h for *Kp*TUAC01 and 12.5 h for *Kp*/pDK-dhaT, respectively. The by-product level was similar between the two strains (data not shown). The most notable difference between the two strains was the 3-HPA accumulated in broth. A 3-HPA peak appeared in both the fermentation broths, but the peak value was 7.55 mmol/L for *Kp*TUAC01 and 1.49 mmol/L for *Kp*/pDK-dhaT.

In the batch fermentation with 50 g/L initial glycerol concentration in the medium, the *Kp*TUAC01 fermentation ceased at 9 h, coincident with the peak of 3-HPA level in broth (12.57 mmol/L). There was also a 3-HPA peak in the broth of *Kp*/pDK-dhaT, but the 3-HPA level was only 2.02 mmol/L, and the fermentation process ran across the 3-HPA peak and continued to use glycerol completely.

Discussion

*Kp*TUAC01 was a 1,3-propanediol-producing strain isolated by our group, it has an excellent 1,3-propanediol productivity in aerobic condition [11]. The sequence of *dhaT* gene cloned from *Kp*TUAC01 has 94% identities to *K. pneumoniae* DSM 2026, which is a widely investigated 1,3-propanediol-producing strain [1]. Though *Kp*TUAC01 and *K. pneumoniae* DSM 2026 belong to the same species, the difference in the nucleotide sequence implied that they have a division in involution. The other two *dhaT* gene homologue strains of *C. freundii* were also 1,3-propanediol-producing strains [6], and the species *C. freundii* and *K. pneumoniae* belonged to the same family of Enterobacteriaceas. In the aspect of coded amino acid sequence, beside the 1,3-propanediol-producing strains *K. pneumoniae* ATCC 25955 *K. pneumoniae* DSM 2026, and *C. freundii* have a high homology to *Kp*TUAC01, a Gram-positive strain *C. pasteurianum* also has a higher homology to *Kp*TUAC01. Though *C. pasteurianum* was a 1,3-propanediol-producing strain [5], it is far from being related to

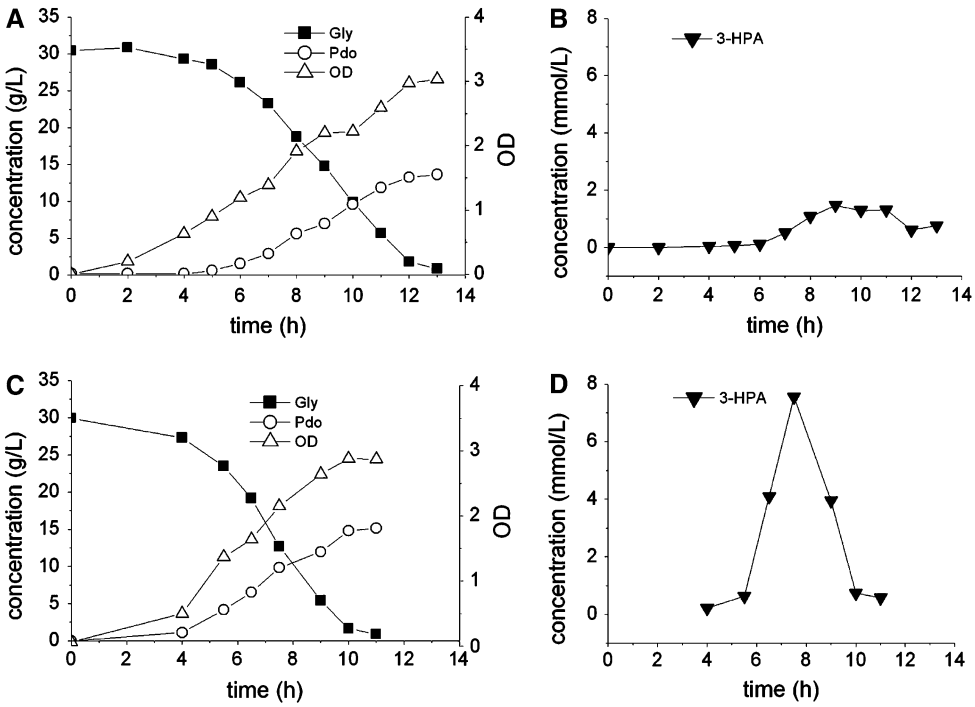

Fig. 5 *Kp*TUAC01 and *Kp*/pDK-dhaT batch fermentations with 30 g/L initial glycerol in medium. (**a**) Curves of substrate consumption, growth, and 1,3-propanediol production of *Kp*/pDK-dhaT fermentation. (**b**) Curves of substrate consumption, growth, and 1,3-propanediol production of *Kp*TUAC01 fermentation. (**c**) 3-HPA level in *Kp*/pDK-dhaT fermentation (peak value: 1.49 mmol/L). (**d**) 3-HPA level in *Kp*TUAC01 fermentation (peak value: 7.55 mmol/L)

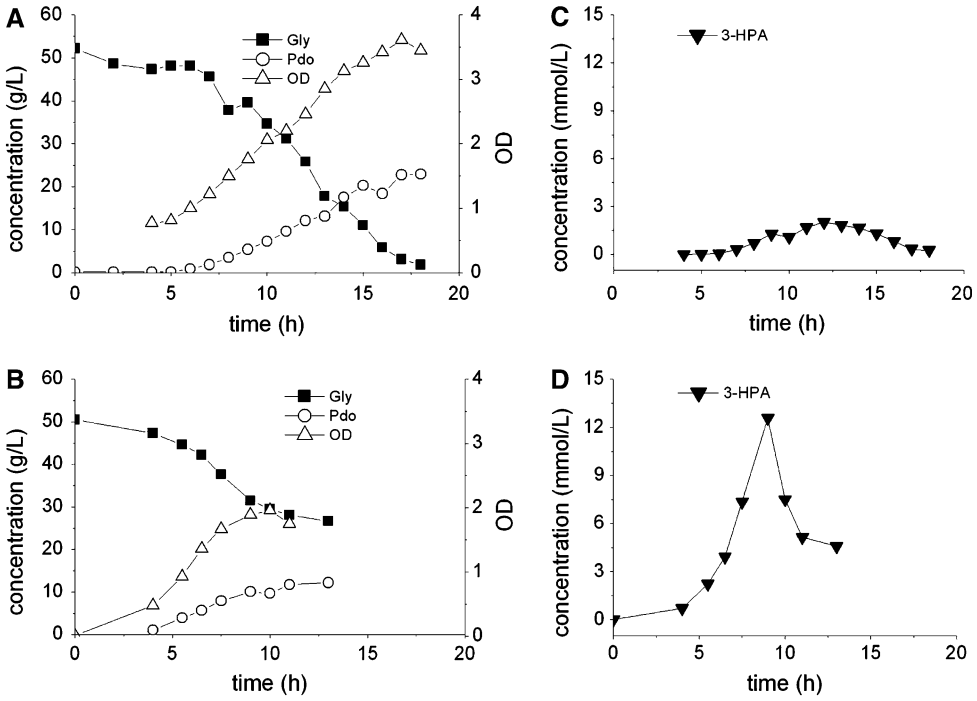

Fig. 6 *Kp* TUAC01 and *Kp*/pDK-dhaT batch fermentations with 50 g/L initial glycerol in the medium. (**a**) Curves of substrate consumption, growth, and 1,3-propanediol production of *Kp*/pDK-dhaT fermentation. (**b**) Curves of substrate consumption, growth, and 1,3-propanediol production of *Kp*TUAC01 fermentation. (**c**) 3-HPA level in *Kp*/pDK-dhaT fermentation (peak value: 2.02 mmol/L). (**d**) 3-HPA level in *Kp*TUAC01 fermentation (peak value: 12.57 mmol/L)

the Enterobacteriacea family in taxonomy. Thus the PDOR shows a high invariance in the protein primary structure, this was dual to the functional requirement.

When cultured in the LB medium, the strain *E. coli*/pDK-dhaT expressed the PDOR irrespective of whether being induced by IPTG or not. In plasmid pDK6, there was a $lacI^Q$ gene which prevents the uncontrolled expression of target gene [12]. The reason for the *dhaT* gene having a low-level expression in *E. coli* is that the *dhaT* gene cloned in this work has 45 upstream nucleotides included; this upstream region contains a *dhaT* gene self-promoter and could be expressed independent of the Ptac promoter.

Strain *Kp*/pDK-dhaT has a similar PDOR expression feature to *E. coli*/pDK-dhaT when cultured in the LB medium. The host strain expressed no PDOR activity but *Kp*/pDK-dhaT expressed the PDOR irrespective of whether being induced by IPTG or not. In the non-induced condition, the expressed PDOR activity was only one-fifth of that in *E.* /pDK-dhaT. This phenomenon was related to the regulation mechanism of *dha* regulon. *dhaT* gene was a construction gene in *dha* regulon; the gene expression in this regulon was induced by dihydroxyacetone (DHA) and glycerol [10]. When there was no inducer, some inhibited regular proteins may bind to the promoter of the regulon and then prevent construction gene transcription. When *Kp*/pDK-dhaT was cultured in LB medium, the inhibitor of the *dha* regulon, coded by the gene in chromosome, may bind in the upstream region of the *dhaT* gene and then limit the gene expression. Whereas no inhibitor gene of the *dha* regulon existed in *E. coli*/pDK-dhaT cell chromosome, *dhaT* gene was expressed for the control of the upstream promoter in the plasmid. For the same regulatory mechanism, the host strain *Kp*TUAC01 does not express *dhaT* gene when cultured in the LB medium.

In flask fermentation, glycerol was presented in the medium, and then *dhaT* gene either in chromosome or in plasmid was expressed. So the strains *Kp*TUAC01 and *Kp*/pDK-dhaT showed PDOR activity in spite of IPTG inducement. *Kp*/pDK-dhaT has a multicopy of the plasmid *dhaT* gene; when induced by IPTG there was a higher PDOR activity. Without being induced, *dhaT* gene in chromosomes and plasmids of *Kp*/pDK-dhaT were also expressed with the presence of glycerol. But the lower PDOR activity in the strain *Kp*/pDK-dhaT than in the host strain *Kp*TUAC01 may contribute to the side effects carried out by the multicopy plasmid.

In flask fermentation, the higher PDOR activity in IPTG-induced *Kp*/pDK-dhaT strain has no effect on the final 1,3-propanediol level. But the effect of the by-products is not clear and has to be studied in future. The changes in conversion ratio of carbon atom in the substrate glycerol to 1,3-propanediol and by products show the PDOR activity has effects on the carbon metabolic flux. The glycerol residue in the broth shows the plasmid pDK-dhaT has an unfavorable effect on the cell metabolism.

In 5 L bioreactor batch fermentation, with 30 g/L initial glycerol in medium, *Kp*/pDK-dhaT has a lower 3-HPA accumulation than *Kp*TUAC01, but the 1,3-propanediol productivity was lower than *Kp*TUAC01. Since the plasmid pDK-dhaT was a multi-copy plasmid, its existence in the

cell might affect the normal grown cell; this was consistent with the flask experiment results. Substrate glycerol was not completely used in *Kp*TUAC01 fermentation with 50 g/L initial glycerol; this was caused by the lethal level of 3-HPA accumulated in broth. While *Kp*/pDK-dhaT could grow normal in this medium, the peak of 3-HPA shown in broth was much lower than that of *Kp*TUAC01. Thus over-expression of the *dhaT* gene in the 1,3-propanediol-producing strain decreased the 3-HPA level in fermentation broth.

Conclusion

The strain *Kp*/pDK-dhaT which over-expressed self *dhaT* gene had the property of reducing 3-HPA accumulation in 1,3-propanediol production. This property gives the strain an advantage in decreasing the hazards of fermentation cessation caused by 3-HPA.

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