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ORIGINAL PAPER

Enhanced 1,3-propanediol production in recombinant *Klebsiella pneumoniae* carrying the gene *yqh*D encoding 1,3-propanediol oxidoreductase isoenzyme

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Abstract The *yqh*D gene from *Escherichia coli* encoding 1,3-propanediol oxidoreductase isoenzyme (PDORI) and the tetracycline resistant gene (*tet*R) from plasmid pHY300 PLK were amplified by PCR. They were inserted into vector pUC18, yielding the recombinant expression vector pUC18-yqhD-tetR. The recombinant vector was then cloned into *Klebsiella pneumoniae* ME-308. The overexpression of PDORI in *K. pneumoniae* surprisingly led to higher 1,3-propanediol production. The final 1,3-propanediol concentration of recombinant *K. pneumoniae* reached 67.6 g/l, which was 125.33% of that of the original strain. The maximum activity of recombinant PDORI converting 3-HPA to 1,3-PD reached 110 IU/mg after induction by IPTG at 31°C during the fermentation, while it was only 11 IU/mg under the same conditions for the wild type strain. The K_m values of the purified PDORI for 1,3-propanediol and NADP were 12.1 mM and 0.15 mM, respectively. Compared with the original strains, the concentration of the toxic intermediate 3-hydroxypropionaldehyde during the fermentation was also reduced by 22.4%. Both the increased production of 1,3-propanediol and the reduction of toxic intermediate confirmed the significant role of 1,3-propanediol oxidoreductase isoenzyme from *E. coli* in converting 3-hydroxypropionaldehyde to 1,3-propanediol for 1,3-PD production.

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Keywords *Klebsiella pneumoniae* · 3-Hydroxypropionaldehyde · 1,3-Propanediol · 1,3-Propanediol oxidoreductase isoenzyme · *yqh*D gene

Abbreviations

PCR	Polymerase chain reaction
IPTG	Isopropyl-β-D-thiogalactopyranoside
1,3-PD	1,3-Propanediol
3-HPA	3-Hydroxypropionaldehyde
GDHt	Glycerol dehydratase
PDOR	1,3-Propanediol oxidoreductase
PDORI	1,3-Propanediol oxidoreductase isoenzyme

Introduction

1,3-Propanediol (1,3-PD) has been considered as an important chemical for a long time. A large-scale application of 1,3-PD is as a monomer in the synthesis of polyesters for use in carpet and textile fibers (Skraly et al. 1998). The conventional chemical method for producing 1,3-PD is rather expensive and generates toxic by-products, and its raw material is derived from nonrenewable petroleum. Microbial production of 1,3-PD is particularly attractive in that it uses renewable resources and reduces pollution to a great extent (Zeng et al. 1993). The 1,3-PD producers are all bacteria including *Klebsiella* (Huang et al. 2002), *Citrobacter* (Boenigk et al. 1993), *Clostridium* (Abbad-Andaloussi et al. 1996) and *Lactobacillus* (Schutz and Radler 1984). Among these microorganisms, *Clostridium* and *Klebsiella* have received more attention because of their relatively high yield and productivity (Zeng and Biebl 1994).

Biosynthesis of 1,3-PD from glycerol is considered as a two-step process in *Klebsiella pneumoniae*. First, glycerol dehydratase (GDHt) (EC 4.2.1.30), encoded by three genes *dha*B, *dha*C and *dha*E, catalyzes the first reaction from glycerol to 3-hydroxypropionaldehyde (3-HPA). Second, 1,3-propanediol oxidoreductase (PDOR) (EC 1.1.1.202), encoded by the *dha*T gene, transfers a reducing equivalent from NADH to 3-HPA, yielding 1,3-PD (Skraly et al. 1998). Both *dha*BCE and *dha*T were expressed successfully in exogenous strains (Tong et al. 1991; Zheng et al. 2006; Tobimatsu et al. 1996), but the activity of PDOR in exogenous strains was far lower than that of GDHt (Zheng et al. 2006; Sun et al. 2003). In the original *K. pneumoniae* strain, similar conditions also existed. As a result, the intermediate 3-HPA was accumulated. Also the activities of GDHt and the growth of the microorganisms may be inhibited by excessive toxic 3-HPA (Zeng and Biebl 2002). Researchers pointed out that any attempts for further metabolic engineering of 1,3-PD production in *K. pneumoniae* should carefully control the accumulation of 3-HPA (Zheng et al. 2006). For this purpose, there are two conceivable approaches including the overexpression of PDOR or weakening the expression of GDHt. However, the exclusion of functional *dha*T gene in recombinant *Escherichia coli* (only harboring gene *dha*BCE) resulted in a significantly higher titer of 1,3-PD (Emptage et al. 2003). Therefore, identification of the non-specific enzyme(s) or gene(s) in native *E. coli* responsible for converting 3-HPA to 1,3-PD may lead to an improved process for the production of 1,3-PD in *K. pneumoiae*. The *yqh*D gene encoding the above non-specific enzyme (alcohol dehydrogenase or PDORI) in *E. coli* was isolated by Emptage et al. (2003). Recently, the completion of the *K. pneumoniae* entire genome sequence has already enabled many functional genomic studies (http://genome.wustl.edu/). This will undoubtly accelerate the developments in the genetic improvement of *K. pneumoniae*. Also they will prove useful for *K. pneumoniae* as an excellent platform host for gene expression in metabolic engineering efforts.

In this study, the *yqh*D gene of *E. coli* JM109 were cloned into *K. pneumoniae* ME-308 to strengthen the catalytic reaction from 3-HPA to 1,3-PD. The inhibitory effect of 3-HPA was reduced. The production and the yield of 1,3-PD were significantly improved. *K. pneumoniae* is resistant to ampicillin, but sensitive to tetracycline and kanamycin. Consequently, replicating plasmids which encode resistance to these latter two antibiotics are potentially useful as cloning vectors for *Klebsiella* strains. Thus the tetracycline resistant gene (tetR) from plasmid pHY300PLK was inserted into the expression vector pUC18 for screening positive colonies.

Materials and methods

Strains, plasmids and culture media

The strains and plasmids used in this study are listed in Table 1. LB medium was used as the seed culture of *E. coli*, *K. pneumoniae* and their derivatives. When *K. pneumoniae* was used for electroporation, EDTA was added to the germ medium to a final concentration of 0.7 mM.

After the electroporation, the seed culture for activating the recombinant *K. pneumoniae* was SOC medium (Joseph and David 2001). When necessary, 10 μg/ml tetracycline was added to the medium as the selection marker.

The fermentation medium for *K. pneumoniae* was: yeast extract 5 g/l, glycerol 40 g/l, $KHPO_4 \cdot 3H_2O$ 10 g/l, KH_2PO_4 2 g/l, NH_4Cl 1 g/l, NaCl 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l,$FeCl_3 \cdot 6H_2O$ 30 mg/l, $CoCl_2 \cdot 6H_2O$ 5 mg/l, VB_{12} 5 mg/l. The fed-batch fermentation was carried out in 3-l bioreactor (NBS, New Brunswick, USA) under the following conditions: medium volume 2 l, inoculation volume 5% (v/v), temperature 31°C, initial pH 7.0, aeration rate 0.4 l/min, and agitation speed 150 rev/min. The glycerol concentration in the medium was maintained at around 25 g/l by manually regulating the feeding rate of the glycerol solution.

Table 1 Bacterial strains and plasmids

Strain and plasmid	Relevant characteristics	Source
Klebsiella pneumoniae ME-308	Host of recombinant plasmid	Lab collection
E. coli DH 5α	Host of recombinant plasmid	Lab collection
E. coli JM109	Source of *yqh*D gene	Lab collection
pUC18	Amp^R	Lab collection
pHY300PLK	Source of Tet^R gene	Lab collection
pMD18- simple-T	Amp^R, 2,692 bp	From Takara company
pMD18- simple-T-yqhD	1.16 kb DNA fragment with *yqh*D gene in T-vector used for sequencing	This work
pUC18-yqhD-tetR	Tet^R, 1.56 kb DNA fragment with *yqh*D gene in pUC18 used for expression	This work

Amp^R, ampicillin resistance; Tet^R, tetracycline resistance

Construction of plasmids and strains

The *yqh*D gene was amplified by PCR using total genome DNA of *E. coli* JM109 as template DNA and primers A1 and A2, which were designed using the sequence information of *yqh*D gene (gene ID 947493, GenBank) from *E. coli* K12.

A1: 5′-*GAATTC*ATGAGCTATCGTATGTTTG-3′
A2: 5′-*GGATCC*CACTCAGAATGCCTGGCGG-3′

The PCR product was purified and connected into cloning vector pMD18-simple-T (products of Takara) for sequencing. After the sequencing, the *yqh*D gene was connected with the expression vector pUC18 using the restriction endonuclease sites *Eco*RI and *Bam*HI that were introduced by the primers used (italicized), yielding plasmid pUC18-yqhD. The tetracycline-resistant gene (tetR) was amplified by PCR using plasmid pHY300PLK as template DNA and primers P1 and P2.

P1: 5′-ACCGGGATCCATGAAATACTGAATTTAAA A-3′
P2: 5′-ACCGAAGCTTCTTAACGATTTAGAAATCC C-3′

The purified PCR product of tetR was then inserted into pUC18-yqhD using the restriction endonuclease sites *Bam*HI and *Hind*III, which were introduced by primers P1 and P2, yielding recombinant plasmid pUC18-yqhD-tetR. Both the PCR programs were performed on thermocycler (Ependdorf) and consisted of: 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 90 s, as well as final extension step of 72°C for 10 min. The PCR mixture consisted of 1 ng each template, 200 mM each dNTP, 20 mM each of the primers, 2.5 µl *Taq* PCR buffer and 0.3 µl Ex *Taq* DNA polymerase (purchased from Takara) in 25 µl. The recombinant plasmid pUC18-yqhD-tetR was purified and transformed into *K. pneumoniae* ME-308 by standard transformation protocol using the method of electroporation (Emptage et al. 2003; Fournet-Fayard et al. 1995).

Preparation of cell-free extracts and assay of the recombinant enzyme activity

IPTG was added into the culture to a final concentration of 1 mM to induce protein expression when recombinant cells grew until the optical density at 600 nm reached 0.4–0.6. The cells were harvested by centrifugation from the fermentation culture. After washing the cell pellet with 0.035 mol/l potassium phosphate buffer (pH 8.0), the cells were resuspended in the same buffer in 3.0 ml. The cells were disrupted ultrasonically at 4°C for 10 min by a cell sonicator (SCIENTZ JY92-II, China). Cell debris was removed by centrifugation, resulting in a cell-free extract, which was subsequently used for the enzyme activity assay.

According to Johnson and Lin (1987), the activity of the enzyme for the reaction of 3-HPA to 1,3-PD can be measured by the formation speed of NADPH from NADP from the reverse reaction due to the instability of 3-HPA and a conversion factor of 3.95 for the forward reaction to 1,3-PD was used according to Ahrens et al. (1998). The reaction was carried out at 25°C in the presence of 0.6 mM NADP, 30 mM $(NH_4)_2SO_4$, 100 mM 1,3-PD and an appropriate volume of the enzyme solution in a 2 ml final K_2CO_3–$KHCO_3$ buffer (pH 9.0). The activity was calculated from the linear slope of increasing absorption of NADPH at 340 nm. One unit of enzyme activity is defined as the amount of protein that produces 1 µmol NADPH per minute. Specific enzyme activity is expressed as units per mg of protein.

Polyacrylamide gel electrophoresis and determination of protein concentration

The SDS–PAGE was conducted on a 12% polyacrylamide gel by the method of Joseph and David (2001). Operation was performed on the Mini-ProteanIII Electrophoresis System (Bio-Rad). Protein on the gel was stained with 0.2% (w/v) Coomassie brilliant blue R-250. Marker proteins with molecular weights ranging from 14.3 to 97.2 kDa were used to estimate the molecular weight of the expression products. The concentration of protein was measured according to the Bradford method with bovine serum albumin (BSA) as standard protein.

Purification of PDORI and measurement of its kinetic properties

After induction of IPTG for 12 h in flasks, the recombinant cells carrying pUC18-yqhD-tetR were harvested, washed with potassium phosphate (20 mM, pH 8.0) buffer twice and suspended in the same buffer at 10% of the volume of harvested culture. The cells were disrupted by sonication at 4°C. Then the mixture was centrifuged at 10,000 rev/min for 10 min. The precipitate obtained was discarded and ammonium sulfate was added to the supernatant to raise the saturation to 50%. After centrifugation, the precipitate was collected and the supernatant was 70% saturated with ammonium sulfate. The 50–70% fraction, which appeared colorless and free of all pigments, was subsequently used for further purification.

The 50–70% fraction was desalted by dialysis and loaded onto a DEAE-Sepharose FF column equilibrated with the potassium phosphate buffer (0.005 mM, pH 8.0). Then it was eluted using a linear gradient 0–0.15 M KCl in 0.005 mM potassium phosphate, pH 8.0. The purified enzyme was used for biochemical characterization. The

values of apparent K_m for the purified enzyme were determined according to Michaelis–Menten equation.

Analysis of biomass and metabolic products

Biomass was determined by measuring the value of optical density at 600 nm with appropriate dilution using a UV–visible spectroscopy system (DU-640, BecKm an, USA). The value of the optical density was converted to cell dry weight (CDW) using a calibration equation ($CDW = 4.183 \times OD_{600} - 0.117$).

Glycerol and 1,3-PD were measured by high-performance liquid chromatography (Summit P 680 HPLC, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; Aminex HPX-87 H Ion Exclusion Column 300 × 7.8 mm, Bio-Rad, USA) under the following conditions: sample volume 10 μl; mobile phase 0.005 M H_2SO_4; flow rate 0.8 ml min^{-1}; column temperature 65°C. 3-HPA was assayed based on the colorimetric method of Circle et al. (1945).

Results and discussion

All values presented in this study are averages of at least three independent trials

Construction of recombinant strains and sequence analysis of yqhD gene

The results of agarose gel electrophoresis of PCR products showed that the two DNA fragments (including the exogenous tetracycline resistance gene) were about 1.16 and 1.56 kb, respectively. The two fragments were inserted into the same plasmid (pUC18), yielding recombinant plasmid pUC18-yqhD-tetR. The recombinant plasmid was transformed into *E. coli* DH5α and *K. pneumoniae* ME-308 successfully. The positive colonies were screened. The recombinant plasmid from the transformed strain was purified and analyzed. Its restriction enzyme identification by agarose gel electrophoresis is shown in Fig. 1, and the structure of recombinant plasmid is shown in Fig. 2.

The amplified *yqh*D gene was connected to plasmid pMD18-simple-T for sequencing. DNA sequence analysis showed that the *yqh*D gene was 1,164 bp, which was in accordance with *E. coli yqh*D gene sequence announced by NCBI Genbank, encoding 387 amino acid residues with a predicted molecular weight of 43 kDa.

SDS–PAGE analysis of protein expression in whole cells

The results of SDS–PAGE analysis of proteins in whole cells are shown in Fig. 3. One protein band about 43 kDa

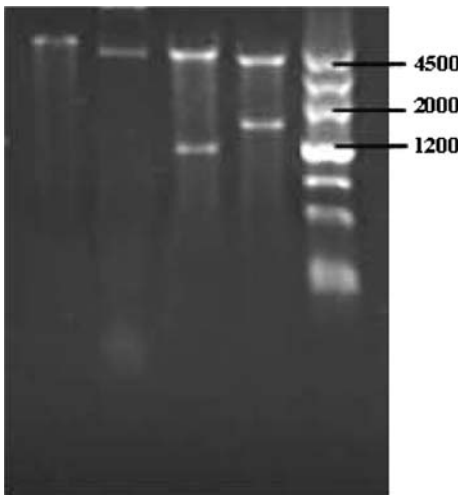

Fig. 1 The restriction enzyme identification of the recombinant plasmid pUC18-yqhD-tetR. 1: Recombinant plasmid digested by *Eco*RI; 2: Circular pUC18-yqhD-tetR plasmid purified from recombinant strain; 3: Recombinant plasmid digested by *Eco*RI and *Bam*HI; 4: Recombinant plasmid digested by *Bam*HI and *Hind*III; 5: DNA marker 4500

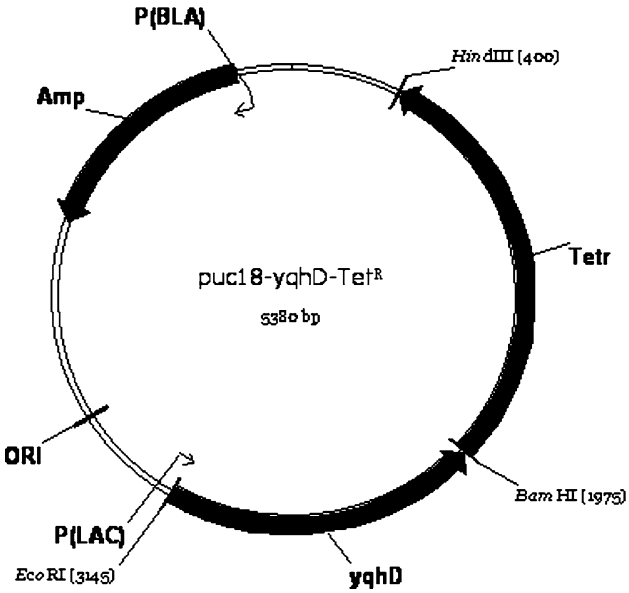

Fig. 2 The structure of the recombinant plasmid pUC18-yqhD-tetR

appeared in the recombinant cells, which corresponded with the prediction from gene sequence. SDS–PAGE also revealed that the target protein of recombinant cells accounted for about 20% of the whole cell proteins under the optimum culture conditions.

Characterization of PDORI

The enzyme was purified after precipitation by ammonium sulfate, desalination by dialysis and loading onto DEAE-Sepharose FF column. The purified recombinant enzyme

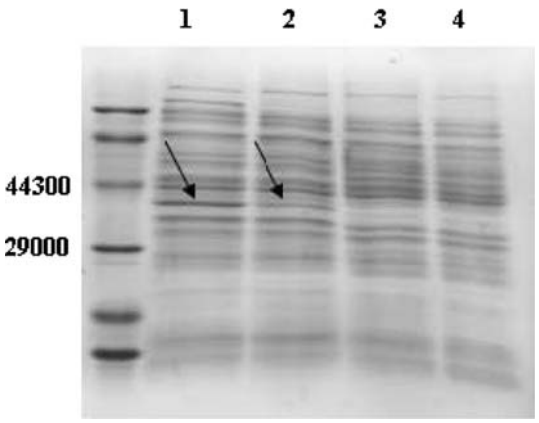

Fig. 3 SDS–PAGE analysis of proteins in whole cells. The *left* is protein marker. 1: *Klebsiella pneumoniae* ME-308 (pUC18-yqhD-tetR)-with IPTG induction; 2: *Klebsiella pneumoniae* ME-308 (pUC18-yqhD-tetR)-without IPTG induction; 3: *Klebsiella pneumoniae* ME-308- with the addition of IPTG; 4: *Klebsiella pneumoniae* ME-308-without the addition of IPTG

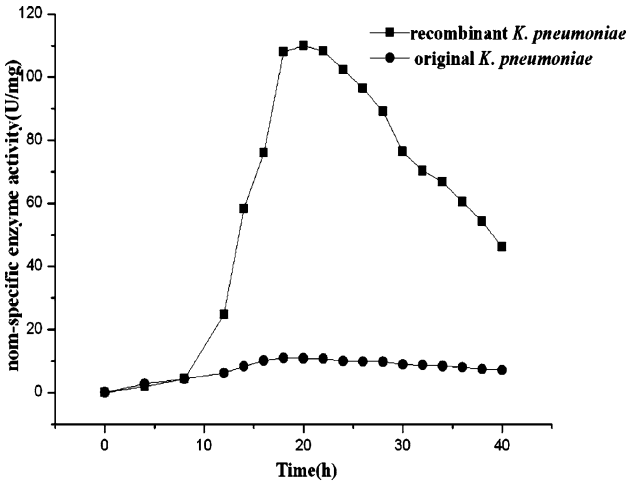

Fig. 4 Time course of the non-specific enzyme activity for the reaction of 3-HPA to 1,3-PD. Enzyme was extracted and assayed from the 3-l bioreactor at different times during the fermentation

exhibited Michaelis–Menten kinetics, and the apparent K_m values for 1,3-PD and $NADP^+$ were 12.1 and 0.15 mM, respectively. The low value of K_m (0.15 mM) of PDORI showed a high affinity for $NADP^+$ to be linked with the enzyme during the reaction between 3-HPA and 1,3-PD, while the value of K_m of native PDOR encoded by gene *dha*T from *K. pneumoniae* was 0.31 mM (Johnson and Lin 1987) for NAD^+ and the value of K_m of recombinant PDOR expressed in *E. coli* was 0.21 mM for NAD^+ (Wang et al. 2005).

Assay of the reaction activity for 3-HPA to 1,3-PD during the fermentation

The high efficiency of the reduction of 3-HPA to 1,3-PD is important during the fermentation of glycerol to 1,3-PD. The expression of PDORI, which catalyzes the non-specific reduction reaction of aldehydes, may strengthen the reaction from 3-HPA to 1,3-PD. As depicted in Fig. 4, the non-specific enzyme activity increased remarkably compared to the control during the fermentation. The maximum enzyme activity (110 IU/mg total protein) for the conversion of 3-HPA to 1,3-PD was tenfold higher than that of the wild type strain. It was supposed that the high activity was contributed to the original expression of *dha*T in *K. pneumoniae* and exogenous expression of *yqh*D from *E. coli*.

Effect of expression of yqhD on cell growth

Growth of the original strain and the recombinant strain in fed-batch cultures was monitored. As shown in Fig. 5, the growth rate (0.16 g/l/h) of the recombinant strain was obviously lower than that of the original strain (0.18 g/l/h) after the IPTG was added. This clearly indicated that the

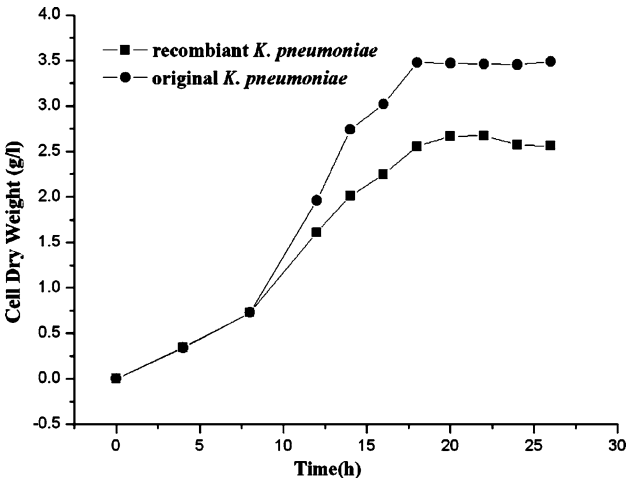

Fig. 5 Time course of cell growth of recombinant *Klebsiella pneumoniae* ME-308 (pUC18-yqhD-tetR) and its parent strain *Klebsiella pneumoniae* ME-308

cell growth of the recombinant strain was impaired by the expression of the exogenous gene. The over-expression of plasmid-encoded protein can be a factor to impose a heavy metabolic burden on the host cell because a large amount of energy is consumed during protein synthesis (Bentley et al. 1990).

Glycerol consumption, 3-HPA formation and 1,3-PD production

Glycerol consumption and 1,3-PD production are described in Fig. 6. Expression of *yqh*D significantly improved 1, 3-PD production. The final titer of 1,3-propanediol achieved 67.6 g/l, which was 125.33% of that of the original strain. Glycerol consumption was 130 g/l, which was also higher

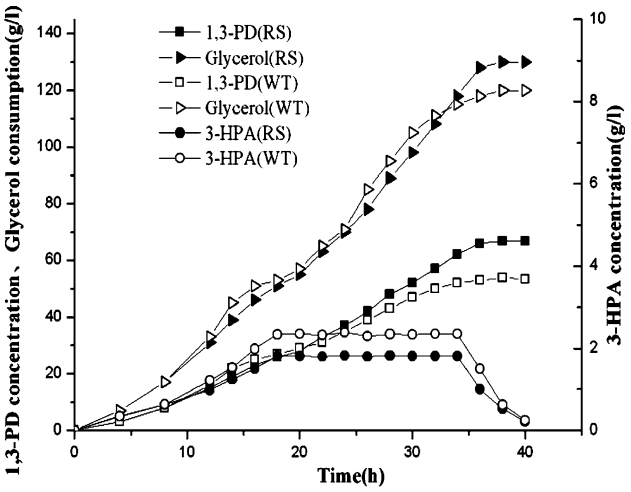

Fig. 6 Time course of 1,3-PD fermentation in 3-l fermentor under micro-aerobic conditions. *RS* recombinant strain; *WT* wild type strain

than that of the control. The amount for the recombinant strain assimilating glycerol was increased by 8.33%. The conversions of glycerol to 1,3-PD were 0.513 and 0.442 g/g, respectively. These demonstrated that the recombinant strain with the expression of PDORI acquired an improved 1,3-PD-producing capacity. Metabolic flux of glycerol in recombinant cells distributed to the 1,3-PD pathway was increased with the expression of PDORI. The specific 1, 3-PD-producing capability (25.98 g/g, 1,3-PD produced per gram of cells) of the recombinant strain was 1.7-fold increased over the original strain (15.28 g/g). The production of 1,3-PD by recombinant *E. coli* only harboring gene *dha*B and *yqh*D was 43.1 g/l (Zhang et al. 2006)under the optimal fermentation conditions, which was far higher than that of the recombinant *E. coli* carrying gene *dha*B and *dha*T constructed before. DuPont had increased the 1,3-PD production to about 135 g/l by recombinant *E. coli* with the *yqh*D gene substituting for the *dha*T gene, including other genetic modifications (Emptage et al. 2003).

The final concentration of 1,3-PD in the fermentation was about 40–60 g/l by the wild-type *K. pneumoniae* without any optimization of the fermentation conditions. Considerable difference about the final product titer exists for different derived *Klebsiella* strains. For example, the production was 70 g/l by *K. pneumoniae* AC 15 isolated from the soil samples with the mathematical optimization method (Zheng et al. 2008), 46.1 g/l by *K. pneumoniae* ATCC 25955, obtained from American type culture collection (Huang et al. 2002) and 72 g/l by *K. pneumoniae* DSM2026, obtained from the German Collection of Microorganisms and Cell Cultures. These are the higher production reported from the papers by the original *K. pneumoniae* strains. In this study, the production of 1,3-PD was improved obviously by recombinants transformed with exogenous gene compared with the original strain. This could encourage the further development of genetic modification of *K. pneumoniae* to produce 1,3-PD efficiently.

Figure 6 also showed the time courses of 3-HPA concentration during the fermentation of glycerol by recombinant *K. pneumoniae* and its derivative strain *K. pneumoniae* ME-308. The 3-HPA concentration of the recombinant strain accumulated to a maximum of around 1.8 g/l after 14 h, which was lower than that of the original strain (2.32 g/l) under the same conditions. Considering the activity of the non-specific enzyme was increased by tenfold, the toxic intermediate 3-HPA accumulation decreased by 22.4%, this also being due to the expression of *yqh*D. As a result, the inhibition on cell growth and on the key enzymes of 1,3-PD metabolic pathway was decreased.

The reason for this phenomenon was that the PDORI encoded by *yqh*D from *E. coli*, which is insensitive to oxygen, can use both NADPH and NADH as its coenzyme (Sulzenbacher et al. 2004). However, the PDOR encoded by gene *dhaT* in native *K. pneumoniae* can only use NADH as its coenzyme. NADH is used for oxidative phosphorylation and as donor of hydrogen for reduction reaction in organisms, while NADPH is mainly used for biosynthesis reduction reaction. Under the aerobic or anaerobic conditions, PDORI could always use both the coenzymes NADPH and NADH to catalyze the conversion 3-HPA to 1,3-PD efficiently. So the amount of toxic intermediate 3-HPA was reduced, also the production and the yield of 1,3-PD was improved significantly.

Conclusion

The *yqh*D gene from *E. coli* was expressed successfully in *K. pneumoniae* ME-308, and the activity of PDORI in the recombinant strain was far higher than that of the original strain.

Compared with the original *K. pneumoniae*, the recombinant strain converts glycerol to 1,3-propanediol with relatively higher production concentration (67.6 g/l) and higher conversion ratio (0.62 mol/mol). And the time achieving the maximum 1,3-PD concentration advanced about 2 h. However, during the first 12 h of the fermentation, the 1,3-propanediol concentration in recombinant *K. pneumoniae* fermentation broth was a slightly lower than that of the original strain. When fermentation time arrived at 8 h, IPTG inducing the expression of gene *yqh*D was added into the fermentation broth, which imposed a metabolic burden and resulted in slowing down the growth rate. After about 20 h, the biomass synthesis of the microorganisms and the expression of *yqh*D achieved the maximum and reached stabilization. Eventually, the 1,3-PD

production of recombinant strain also achieved the maximum. The results confirmed the expression of *yqh*D as a positive factor in the metabolic pathway for 1,3-PD production.

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