

# Overexpression of genes of the *dha* regulon and its effects on cell growth, glycerol fermentation to 1,3-propanediol and plasmid stability in *Klebsiella pneumoniae*

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## Abstract

Glycerol dehydratase that catalyses the conversion of glycerol to 3-hydroxypropionaldehyde was previously supposed to be a limiting step in the 1,3-propanediol production from glycerol. In this study, glycerol dehydratase was over-expressed separately or coordinately with 1,3-propanediol oxidoreductase in *Klebsiella pneumoniae* DSM2026 to investigate its effects on the glycerol fermentation. The overexpression of glycerol dehydratase surprisingly led to a serious impairment of growth of *K. pneumoniae* in both continuous and batch cultures. Instability of the plasmids bearing the genes encoding glycerol dehydratase and/or 1,3-propanediol oxidoreductase was observed in recombinant cells, especially in anaerobic cultures using glycerol as sole carbon source. It is postulated that an imbalanced conversion of glycerol to the intermediate 3-hydroxypropionaldehyde and its further conversion to 1,3-propanediol and the associated accumulation and toxicity of 3-hydroxypropionaldehyde are responsible for the phenomena observed. Furthermore, the putative regulatory gene *dhaR* of the *dha* regulon was also overexpressed in *K. pneumoniae*. The increased expression of 1,3-propanediol oxidoreductase confirmed the role of DhaR as a positive regulator of the *dhaT* gene. © 2006 Elsevier Ltd. All rights reserved.

**Keywords:** Glycerol metabolism; Glycerol dehydratase; 1,3-Propanediol oxidoreductase; 3-Hydroxypropionaldehyde; *DhaR*; *Klebsiella pneumoniae*

## 1. Introduction

Anaerobic glycerol metabolism is of fundamental importance for the microbial production of 1,3-propanediol (1,3-PD). Genes corresponding to anaerobic glycerol metabolism are typically organized as a gene cluster called *dha* regulon. It has been identified in natural 1,3-PD producers such as *Klebsiella pneumoniae* [1,2], *Citrobacter freundii* [3], *Clostridium pasteurianum* [4,5] and *Clostridium butyricum* [6]. Due to the industrial interest on using 1,3-PD as a monomer for a novel polymer polytrimethylene terephthalate (PTT), a glucose-utilizing strategy by metabolically engineered *E. coli* strain expressing a glycerol 3-phosphate dehydrogenase (DAR1) and glycerol 3-phosphate phosphatase (GPP2) genes from *Saccharomyces cerevisiae* and the *dhaB* genes of *K. pneumoniae*

was developed by Genencor and Dupont [7]. In Europe, the glycerol-utilizing process is of particular interest due to the abundance of glycerol as a by-product in the biodiesel production from rapeseed oil.

In the model organism *K. pneumoniae* for glycerol metabolism it primarily involves two branch pathways: the reductive branch and the oxidative branch. 1,3-PD is produced by the reductive branch in two successive enzymatic reactions. Glycerol dehydratase (GDHt), encoded by three genes *dhaB*-alpha, *dhaB*-beta and *dhaB*-gamma, catalyzes the first reaction from glycerol to 3-hydroxypropionaldehyde (3-HPA) [8,9]. The latter is then reduced to 1,3-PD by the enzyme 1,3-propanediol oxidoreductase (PDOR) encoded by the gene *dhaT* under the consumption of reducing power NADH<sub>2</sub> [1,10]. The initial steps of the oxidative pathways are catalyzed by the enzymes glycerol dehydrogenase and dihydroxyacetone kinase. The enzymes GDHt and PDOR obtained from *K. pneumoniae* have been overexpressed in *E. coli* [2,11] and 6.3 g/L of 1,3-PD was produced in a fed-batch fermentation

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using glucose and glycerol as co-substrates. Kinetic and pathway analysis suggested that GDHt is a major rate-limiting enzyme for the consumption of glycerol and for the formation of 1,3-PD in *K. pneumoniae*, especially at high glycerol concentrations [12]. It has also been shown that GDHt activity is a limiting step for 1,3-PD production in *C. butyricum* [13]. Therefore, increasing the dehydratase activity in the native producer *K. pneumoniae* could probably increase the productivity of 1,3-PD fermentation.

DhaR, encoded by the gene *dhaR* in the *dha* operon, shows a high similarity to the AAA<sup>+</sup> (ATPases associated with various cellular activities) superfamily of enhancer binding proteins, consisting of a N-terminal sensing domain, a central structurally conserved ATPase domain and a C-terminal helix-turn-helix motif. It was suggested to be a transcriptional activator due to its role on the up-regulation of glycerol dehydrogenase in *C. freundii* [3]. Its close homolog in *E. coli* has been recently shown to stimulate the transcription of the *dhaKLM* operon (corresponding to the *dhaK* operon in *K. pneumoniae* [1]) from a sigma 70 promoter and its transcription is negatively auto-regulated [14]. Genetic and biochemical studies indicate that the enzyme subunits DhaL and DhaK act antagonistically as coactivator and corepressor of the transcription activator by mutually exclusive binding to the sensing domain of DhaR [14]. It is interesting to investigate its putative role on the expression of enzymes of the reductive pathway of 1,3-PD production.

In this study, the effects of overexpression of *dhaB* (solely or together with *dhaT*) and *dhaR* on the cell growth and glycerol fermentation in *K. pneumoniae* DSM2026 were studied. The stability of plasmids in the different recombinants and under different fermentation conditions was also examined. The results lead to a better understanding of many experimentally observed phenomena of the glycerol fermentation and have important implications for developing better strategies for the metabolic engineering of 1,3-propanediol production.

## 2. Materials and methods

### 2.1. Bacterial strains, media, and growth conditions

Bacterial strains and plasmids used in this study are given in Table 1. LB medium was used as a rich medium for the routine growth of *K. pneumoniae* strains. The composition of the continuous culture and batch culture media for

*K. pneumoniae* was described previously [15,16]. The initial glycerol concentration for the continuous culture was 30 g/L and the concentration in the feed medium was 50–175 g/L. Around 100 g/L glycerol was used in the batch cultivation. For the cultivation of *K. pneumoniae* 150 µg/mL carbenicillin (Cb) plus 100 µg/mL ampicillin (Ap) were used in flask culture while 200 µg/mL Cb plus 133 µg/mL Ap were used in the continuous or batch cultures.

Shake flask cultivation were used for seed culture, initial investigation of the overexpression of GDHt and PDOR by different plasmids and the study of plasmid stability in variant conditions. If not specified, anaerobic flask cultivation was performed in septum bottles at 37 °C using medium containing 20 g/L glycerol described elsewhere [17].

For continuous culture and batch culture a 4 L Setric Bioreactor (Set 4V, Setric Genie Industrial, Toulouse, France) with a working volume of 2 and 3 L was used, respectively. The reactor was connected to a real-time computer control system (UBICON, ESD, Hannover, Germany) for on-line data acquisition. All cultivations were carried out at 37 °C, pH 7.0 and 300 rpm. To ensure anaerobic condition, the bioreactor was sparged with nitrogen at a flow rate of 0.4 vvm. The cultivation strategy was described previously by Menzel et al. [16].

### 2.2. DNA manipulations

General DNA manipulations were performed as described by Sambrook and Russel [18]. Plasmids were isolated with the plasmid purification kit Jetstar (Genomed, Bad Oeynhausen, Germany). Genomic DNA from *K. pneumoniae* was extracted with a DNA Kit (Qiagen, Hilden, Germany). DNA fragments were recovered from gels with the Jetsorb kit (Genomed, Bad Oeynhausen, Germany). Electroporation of *K. pneumoniae* was done with the gene pulser II (BioRad, Germany) at 2.5 kV, 50 µF, and 150 Ω by using a 2 mm cuvette. T4 DNA-polymerase and restriction enzymes (Promega, Madison, USA) were used as recommended by the suppliers. DNA sequencing was performed by using the ABI PRISM Dye Terminator (Perkin-Elmer, Weiterstadt, Germany).

### 2.3. Construction of plasmids pKM13, pKM15 and pKM16

To construct the plasmid pKM13, the plasmid pTC3 [19] bearing the *dha*-regulon (*dhaB*, *dhaT*, *dhaD*, *dhaK* and *dhaR* gene) from *Klebsiella* was digested with *KpnI* and the resultant sticky end was treated with T4-DNA polymerase to create a blunt end. A second digestion was performed with *MluI* and a 3.343 kb fragment containing the intact *dhaR* and partial *dhaT* (3'-end) genes was isolated for further construction. The plasmid pTC18 [20], a derivative of pTC3, was digested with *SalI*, and the end was filled with Klenow polymerase. The created fragment was then digested with *MluI*. A 10.664 kb big fragment containing the intact *dhaB* and partial *dhaT* (5'-end) genes was recovered from gel and ligated with the 3.343 kb fragment, generating a plasmid named as pKM13 (14.007 kb) (Fig. 1). It contains the *dhaB*, *dhaT* and *dhaR* genes from *K. pneumoniae* ATCC 25955.

Plasmids pKM15 and pKM16 are derivatives of pKM13. For this purpose, the plasmid pKM13 was double digested with *KpnI* and *MluI* and a 1.224 kb small fragment containing the partial *dhaT* gene was removed. The rest big fragment was treated with T4-DNA polymerase and was then self-ligated. The created plasmid was designated as pKM15 (12.783 kb) (Fig. 1), containing the

Table 1  
Bacterial strains and plasmids used in this study

Strains or plasmids	Genotype/phenotype	Reference
<b>Strains</b>		
<i>K. pneumoniae</i> DSM2026	Wild type	DSMZ, Germany
<b>Plasmids</b>		
pBR322	Cloning vector, Ap <sup>r</sup> , Tc <sup>r</sup>	[42]
pTC3	Derivative of plasmid pBR322, bearing <i>K. pneumoniae</i> <i>dhaB</i> , <i>dhaT</i> , <i>dhaD</i> , <i>dhaK</i> gene	[19]
pTC18	pTC3 without <i>SalI</i> – <i>SacI</i> fragment	[20]
pKM13	pTC3 without <i>SalI</i> – <i>KpnI</i> (10.06 kb) fragment, bearing <i>K. pneumoniae</i> <i>dhaB</i> , <i>dhaT</i> , <i>dhaR</i> gene	This study
pKM15	pKM13 without <i>KpnI</i> – <i>MluI</i> fragment, bearing <i>K. pneumoniae</i> <i>dhaB</i> , <i>dhaR</i> gene	This study
pKM16	pKM13 without <i>AatII</i> – <i>MluI</i> (6.794 kb) fragment, bearing <i>K. pneumoniae</i> <i>dhaR</i> gene	This study

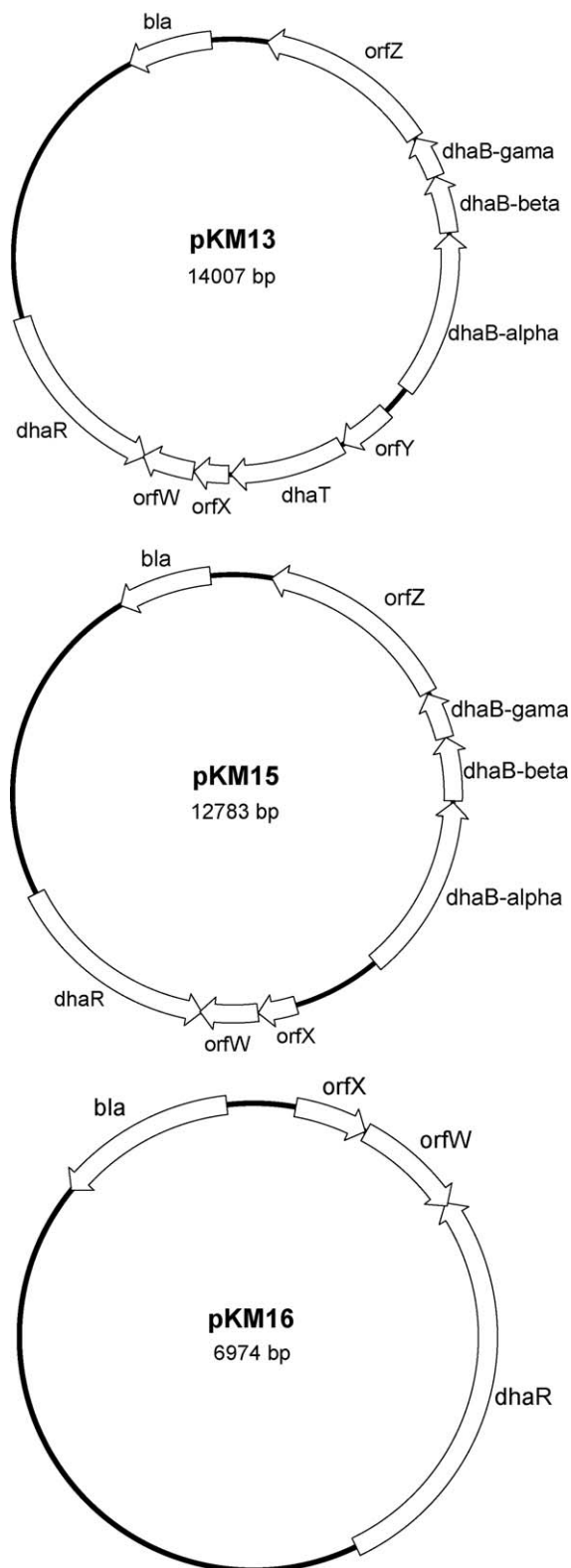


Fig. 1. Plasmids pKM13, pKM15 and pKM16.

*dhaB* and *dhaR* genes. The plasmid pKM16 (Fig. 1) was generated as follows. The plasmid pKM13 was double digested with *AatII* and *MluI* and a 6.974 kb big fragment containing only the intact *dhaR* gene was recovered. Both ends of the fragment were treated with T4-DNA polymerase and were then self-ligated.

#### 2.4. Analysis of biomass, substrate and products

The biomass concentration was measured as absorbance at 650 nm and as dry weight (grams dry weight per liter) as well. The concentrations of the products ethanol, acetate and 1,3-PD were measured with gas chromatography (Chrompack 483A) as described elsewhere [16]. Glycerol and lactate were determined using enzymatic test kits from Boehringer (Mannheim, Germany).

#### 2.5. Enzyme assays

Culture samples for enzyme assays were taken anaerobically and cell-free extracts were prepared as described previously [15]. Protein concentration was determined according to Lowry et al. [21]. The activity of 1,3-propanediol oxidoreductase was determined using the reverse reaction (1,3-PD conversion to 3-HPA) due to the instability of 3-HPA, as described by Forage and Foster [22] and a conversion factor of 3.95 for the physiological reaction was used according to Ahrens et al. [12]. The activity of glycerol dehydratase was determined as described by Ahrens et al. [12] and a correction factor of 1.41 was used.

One unit of enzyme activity is defined as the amount of enzyme required to reduce 1  $\mu$ mol of substrate per minute. Specific enzyme activity is expressed as units per mg of protein.

#### 2.6. Southern blot analysis

The *NotI* and *HindIII* digested genomic DNA of *K. pneumoniae* and digested plasmids pKM13 and pKM15 were separated by electrophoresis and then transferred to a positively charged nylon membrane Hybond-N (Amersham, Buckinghamshire, England). The DNA was cross-linked to the nylon membrane with a UV Stratalinker (Stratagene, California, USA). A *dhaB*-specific probe (an *AatII*–*NotI* fragment of pTC3) and a *dhaR*-specific probe (a *SacI*–*KpnI* fragment of pTC3) were labelled with  $^{32}$ P by using a “Prime a gene” kit (Promega, Madison, USA). The hybridisation was performed by following the recommendation from the supplier (Amersham, Buckinghamshire, England). The membrane was exposed to the Kodak Biomax film overnight.

### 3. Results

#### 3.1. Overexpression of glycerol dehydratase and 1,3-propanediol oxidoreductase in flask cultures

The plasmids pKM13, pKM15 and pKM16 (Fig. 1) carrying different genes of the *dha* regulon from *Klebsiella* were constructed as described in Section 2 and transformed into *K. pneumoniae* DSM2026.

The expression data of the corresponding enzymes in anaerobic shake flask cultivations with glycerol as sole carbon source are summarized in Table 2. In the strains harboring pKM13 or pKM15, the specific activity of GDHt was 137- and 27-fold higher than that in the wild type strain, respectively,

Table 2

Overexpression of glycerol dehydratase (GDHt) and 1,3-propanediol oxidoreductase (PDOR) in recombinant *K. pneumoniae* DSM2026 in anaerobic shake flasks

Strain carrying plasmid	GDHt ( <i>dhaB</i> ) (U/mg protein)	PDOR ( <i>dhaT</i> ) (U/mg protein)
–	0.278 $\pm$ 0.052	0.316 $\pm$ 0.038
pKM13	38.063 $\pm$ 0.823	14.559 $\pm$ 1.100
pKM15	7.575 $\pm$ 0.535	0.428 $\pm$ 0.030

A preculture minimal medium containing 20 g/L glycerol [17] was used and the cultivation was performed at 37  $^{\circ}$ C in septum bottles.

whereas PDOR was over-expressed by 46-fold higher in the strain bearing pKM13 than that in the wild type, clearly indicating a successful overexpression by increasing the gene dosage. Further quantitative experiments were done to verify the effects of overexpression of these genes on the production of 1,3-PD.

### 3.2. Enzyme expression in continuous culture by engineered recombinants

Our preceding experiment with a *K. pneumoniae* recombinant harbouring a plasmid pTC53 containing *dhaB* and *dhaT* under the control of IPTG-inducible *trc* promoter showed that the plasmid was stable up to 25 generations in continuous culture at a dilution rate  $0.1 \text{ h}^{-1}$  [23]. Continuous cultivations of the wild type and the recombinant strains of DSM2026 (pKM13) and DSM2026 (pKM15) were therefore carried out under similar conditions in this study. Five steady states were reached successively by variation of the glycerol concentration in the feeding medium. It took at least 40 h to reach a steady-state at a dilution rate of  $0.1 \text{ h}^{-1}$ . For instance for the wild type strain, after 7 h of batch culture a feeding of medium with 50 g/L glycerol was initiated. The first steady state was reached at 49.5 h of the cultivation and 3.14 g/L biomass and 202.82 mM 1,3-PD were achieved. The biomass yield and the 1,3-PD yield were 0.062 g/g and 0.306 g/g, respectively. The concentrations of other products such as ethanol, acetic acid, succinic acid and lactic acid at the first steady state were 193.34, 77.49, 5.93 and 1.31 mM, respectively, and the corresponding production yield were 0.176, 0.092, 0.014 and 0.002 g/g, respectively. Enzyme assays were done and the results are partly shown in Fig. 2. Surprisingly, we could not find any significant increase in the expression of GDHt and PDOR in both recombinant strains as

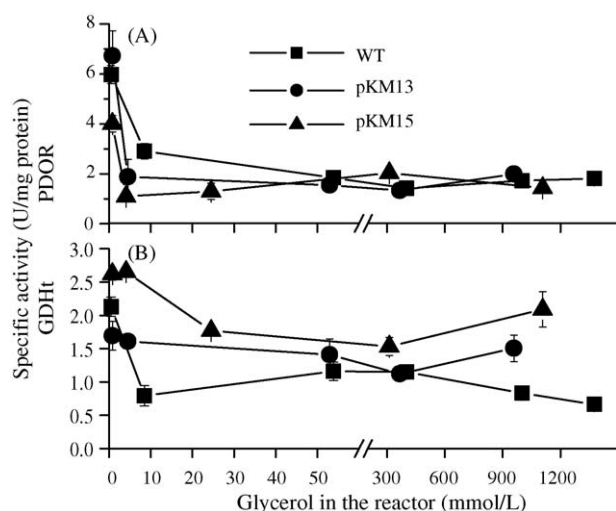


Fig. 2. Specific activity of (A) 1,3-propanediol oxidoreductase (PDOR) and (B) glycerol dehydratase (GDHt) of the wild type and the recombinant strains DSM2026 (pKM13) and DSM2026 (pKM15) in continuous cultures. A minimal medium containing 30 g/L initial glycerol and a feed medium containing 100 g/L glycerol were used for cultivations in a 4 L computer controlled bioreactor with a working volume of 2 L. All cultivations were carried out at 37 °C, pH 7.0 and 300 rpm.

observed from shake flask cultivations mentioned above. The expression of other enzymes related with the consumption of glycerol, such as glycerol dehydrogenase, dihydroxyacetone kinase, pyruvate kinase, citrate synthase and pyruvate dehydrogenase, and the production of 1,3-PD in the recombinants were also measured. However, none of them exhibited any significant difference in comparison with the wild type (data not shown).

We suspected that it was due to the loss of the plasmids or the inserts. To test this assumption, we examined the presence of the inserts by using southern blot analysis with probe specific to *dhaB* or to *dhaR*. Samples were taken from different steady states of the continuous cultures and also from anaerobic flask cultures of the recombinant strains *K. pneumoniae* DSM2026 (pKM13) or DSM2026 (pKM15). Since pKM13 and pKM15, which are both pBR322-based plasmids, are expected to have a medium copy number per cell, the hybridization signal corresponding to the vector should be much stronger than that to the chromosomal DNA, if the inserts or the plasmids are not lost. The bands for the samples from flask cultures were close to this expectation (Fig. 3). However, the *dhaB* gene was completely lost at all steady states of the recombinant harbouring the plasmid pKM15. In the recombinant containing pKM13, the *dhaB* gene was also seriously lost, especially when the glycerol concentration was increased (from steady states 1–5). Similar results were observed when a *dhaR*-specific probe was applied [23].

### 3.3. Expression study in batch cultivation

The successful overexpression of GDHt and PDOR (Table 2) and no plasmid loss showed by southern blot analysis (Fig. 3) in flask cultures suggest that batch cultivation could be a more feasible strategy for the metabolic engineering of 1,3-PD production. Anaerobic batch cultivations in the computer-controlled bioreactor for both the wild type and the recombinant strain DSM2026 (pKM13) using glycerol as sole carbon source were thus carried out to investigate the behaviour of these recombinants. Antibiotics (200  $\mu\text{g/mL}$  Cb plus 130  $\mu\text{g/mL}$  Ap) were supplied in the medium for the recombinant before inoculation. The preculture was prepared in an anaerobic shake flask using glycerol as sole carbon source.

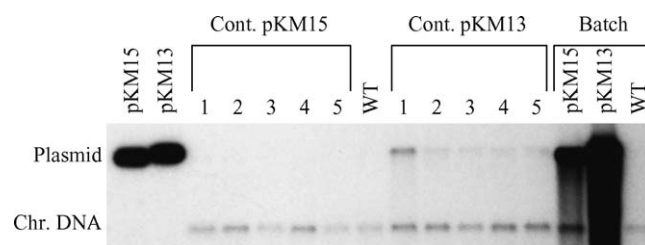


Fig. 3. Southern blot analysis of plasmid stability in continuous culture and anaerobic flask culture of the recombinant strains *K. pneumoniae* DSM2026 (pKM13) and DSM2026 (pKM15). The probe is specific to the *dhaB* gene. Numbers 1–5 indicates samples taken from different steady states from substrate limitation to substrate excess. Plasmids pKM13, pKM15 and the chromosome DNA of the wild type are used as positive controls.

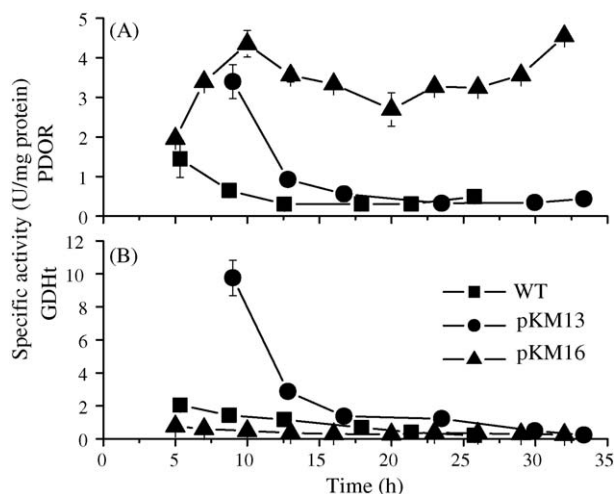


Fig. 4. Specific activity of (A) 1,3-propanediol oxidoreductase (PDOR) and (B) glycerol dehydratase (GDHt) in the batch cultivations of the wild type and recombinant strains of *K. pneumoniae* DSM2026, DSM2026 (pKM13) and DSM2026 (pKM16). A minimal medium containing 100 g/L glycerol was used for cultivations in a 4 L computer controlled bioreactor with a working volume of 3 L. All cultivations were carried out at 37 °C, pH 7.0 and 300 rpm.

In agreement with the results of flask cultures, pKM13 recombinant showed significant overexpression of GDHt and PDOR, resulting in an up to five-fold increase of the activities at the beginning of the cultivation (Fig. 4). Both GDHt and PDOR activities reduced rapidly during the prolonged cultivation. After 17 h, the activities of both enzymes in the recombinant dropped down to the same level as that in the wild type. A delayed consumption of glycerol and an extended lag growth phase (Fig. 5A) were observed in the cultivation of the recombinant, implying a metabolic burden placed on the host by the plasmid. The maximum biomass concentration, the overall biomass yield and  $\mu_{\max}$  were 2.61 g/L, 0.024 g/g and 0.7 h<sup>-1</sup> for the wild type strain and 2.27 g/L, 0.020 g/g and 0.48 h<sup>-1</sup> for the recombinant strain DSM2026 (pKM13), respectively. The initial overexpression of the enzymes at the early stage of the process did not lead to an enhanced production of 1,3-PD (Fig. 5B). The production profiles of 1,3-PD, ethanol and acetate in the recombinant were all similar to the corresponding ones in the wild type except for a delay of several hours (Fig. 5B). The maximum concentration and the yield of 1,3-PD were 656.23 mmol/L and 0.462 g/g for the wild type strain, and 656.57 mmol/L, 0.465 g/g for the recombinant strain DSM2026 (pKM13) respectively. Interestingly, the initiation of rapid production of 1,3-PD and fast cellular growth seemed to be correlative with the decline of the enzyme activity of GDHt and PDOR (Figs. 4 and 5). Considering the results of the continuous cultures, we may also suspect problems of plasmid stability during batch fermentation.

### 3.4. Plasmid stability

Samples from batch cultures were plated on LB agar plates with or without antibiotics (200  $\mu$ g/mL Cb and 130  $\mu$ g/mL Ap) to check the plasmid stability. No colony grew up on the selective agar plate from the samples of DSM2026 (pKM13)

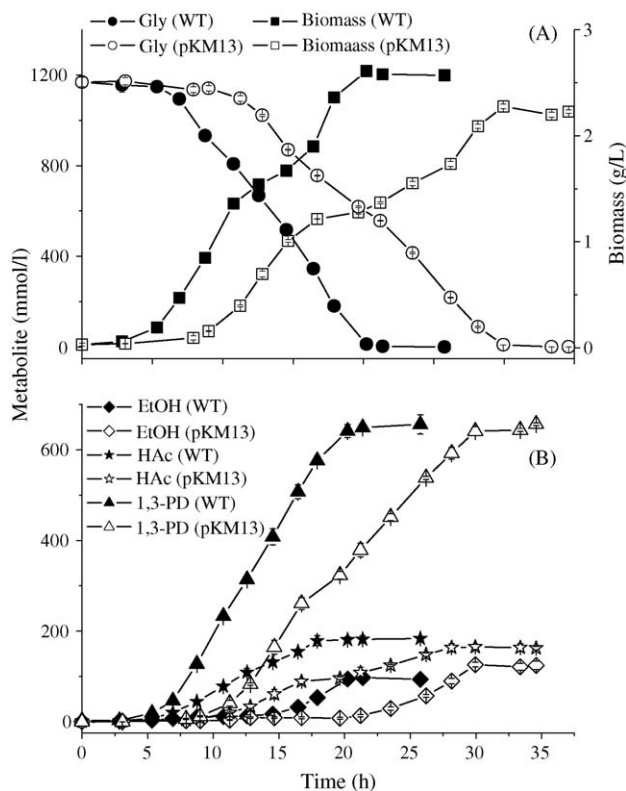


Fig. 5. Batch cultivations of the wild type *K. pneumoniae* DSM2026 and the recombinant strain DSM2026 (pKM13). The cultivation conditions was the same as described in Fig. 4. (A) Glycerol consumption and biomass. (B) Products. Gly, EtOH, HAc and 1,3-PD are abbreviations for glycerol, ethanol, acetate and 1,3-propanediol, respectively.

culture after 10 h of batch cultivation. To exclude a possible impurity in the stock of the strain the transformation of pKM13 was repeated. The new transformants were confirmed by plating and plasmid extraction. A batch cultivation was then repeated with the newly-constructed recombinant. Again, all the cells could grow up on LB agar plates but not on the selective plates. Since all the cells remain in the bioreactor, these results would be hardly understood unless the cells harboring plasmids were seriously damaged.

To test this hypothesis, four DSM 2026 recombinants carrying freshly transformed plasmid pKM13, pKM15, pKM16 and pBR322, respectively, were cultivated in shake flasks under different combinations of conditions: aerobically or anaerobically, in a medium with glycerol as sole carbon source or in a rich LB medium, with or without antibiotics (Cb and Ap) (Table 3). Plasmid pBR322 was used as a control since all the other three plasmids are originated from pBR322. Samples from 12 h cultures were diluted to 10<sup>-5</sup>- and 10<sup>-6</sup>-fold and spread on agar plates with or without antibiotic pressure for another 12 h cultivation. The plasmid stability was shown roughly as the ratio in percentage of colonies on the antibiotic agar plates over that on the agar plates without antibiotics. The results showed that all the four plasmids are stable in the aerobic cultures even without antibiotic pressure. The plasmids pKM16 (*dhaR*) and pBR322 are also stable in the anaerobic cultures and the stability is independent of the antibiotic pressure and the

Table 3

The stability of plasmids pKM13 and pKM15 in *K. pneumoniae* DSM2026 was significantly affected by the genes harbored and by the cultivation conditions

Plasmids	Genes on plasmid	Combination of cultivation conditions							
		+O <sub>2</sub> , Glyc, +ApCb	+O <sub>2</sub> , Glyc, -ApCb	+O <sub>2</sub> , LB, +ApCb	+O <sub>2</sub> , LB, -ApCb	-O <sub>2</sub> , Glyc, +ApCb	-O <sub>2</sub> , Glyc, -ApCb	-O <sub>2</sub> , LB, +ApCb	-O <sub>2</sub> , LB, -ApCb
pKM13	<i>dhaR, dhaB, dhaT</i>	79.7 ± 5.1	107.7 ± 3.6	96.5 ± 1.6	101.3 ± 0.5	50 ± 3.6	17.8 ± 0.3	98 ± 3.0	50 ± 1.4
pKM15	<i>dhaR, dhaB</i>	78 ± 2.2	82.9 ± 4.2	90.6 ± 4.4	94.9 ± 3.1	0 ± 0	0 ± 0	56.1 ± 1.4	53.7 ± 2.1
pKM16	<i>dhaR</i>	101 ± 1.5	90.6 ± 5.1	97.3 ± 1.8	83.9 ± 0.4	101.5 ± 1.2	87.3 ± 1.4	71.2 ± 0.6	98.9 ± 1.1
pBR322	–	124 ± 1.8	116.6 ± 2.4	89.3 ± 2.3	94 ± 2.1	71.4 ± 0.7	86.2 ± 2.2	100 ± 0.8	111 ± 0.9

The number shows the ratio in percentage of colonies on agar plates containing antibiotics to colonies on agar plates without antibiotics. ±O<sub>2</sub>: aerobic or anaerobic; ±ApCb: with or without antibiotics (Ap 100 µg/mL, Cb 150 µg/mL); Glyc/LB: glycerol as sole carbon source or LB medium.

media. However, pKM15 and pKM13 are unstable in the anaerobic cultures, especially when glycerol was used as sole carbon source. The plasmid pKM15 (*dhaB*) was completely lost in the anaerobic cultures using glycerol as sole carbon source even though the antibiotics was added. The plasmid pKM13, harboring *dhaT* in addition to *dhaB*, showed a less serious loss (50% and 17.8% of the cells still harbored the plasmid pKM13 in the anaerobic cultures with glycerol as sole carbon source with or without antibiotic pressure, respectively). This is consistent with the results in the continuous cultures. An unexpected loss of the plasmids on the LB agar plate (more than 40% of pKM15 in the cultures with or without antibiotics and 50% of pKM13 in the case of no antibiotics) was probably due to the rest glycerol (0.4 g/L) from the stock culture. These comparative results clearly indicate that the unbalanced overexpression of *dhaB* causes the instability of the plasmids.

### 3.5. Effect of overexpression of *dhaR*

The *dhaR* gene from *K. pneumoniae* ATCC25955 was cloned and sequenced. The sequence was submitted to the Genbank and the accession number is DQ402048.

As mentioned in the introduction section, DhaR is a transcription factor activator and responsible for the induction of the expression of two enzymes in the oxidative pathway (DHAK in *E. coli* and GDH in *C. freundii*) [3,14]. The involvement of DhaR in the regulation of *dha* regulon of *K. pneumoniae* is generally unknown. It is of interest to investigate if it has any positive effect on the enzymes involved in the reductive pathway of glycerol fermentation in *K. pneumoniae*. To this end, *K. pneumoniae* DSM2026 bearing the plasmid pKM16 was cultivated in batch fermentation using the same medium as before (Figs. 4 and 6). The overall level of PDOR activity was greatly enhanced up to 6.7-fold during the fermentation in the recombinant than that in the wild type (Fig. 4), indicating that DhaR positively regulates the expression of PDOR. The activity of GDHt, however, was reduced.

A promoted growth and an increased consumption of glycerol were observed in the early growth phase (Fig. 6A). The growth ceased suddenly after 10 h of cultivation and recovered to some extent after another 10 h. The maximum biomass, the global biomass yield and  $\mu_{max}$  for the wild type strain and the recombinant strain DSM2026 (pKM13) were 2.61 g/L versus 1.83 g/L, 0.024 g/g versus 0.018 g/g and 0.7 h<sup>-1</sup> versus

0.4 h<sup>-1</sup>, respectively. The recombinant carrying the plasmid pKM16 was shown to produce maximum 440 mM 1,3-PD (Fig. 6A), unexpectedly low compared to that in the wild type (656.23 mM) even though PDOR was significantly up-regulated. The yield of 1,3-PD for the wild type strain and the recombinant strain DSM2026 (pKM16) were 0.462 g/g versus 0.338 g/g, respectively. The maximum amount of acetate (141.2 mM) (Fig. 6B) produced by the recombinant was only three fourth of that produced by the wild type. The production of lactate was accelerated after 10 h of fermentation, whereas the production rate of 1,3-PD was apparently reduced at the same time (Fig. 6A and B), indicating that sufficient NADH<sub>2</sub> was channeled to the production of lactate. The maximum amount of lactate for 105.7 mM was achieved by the

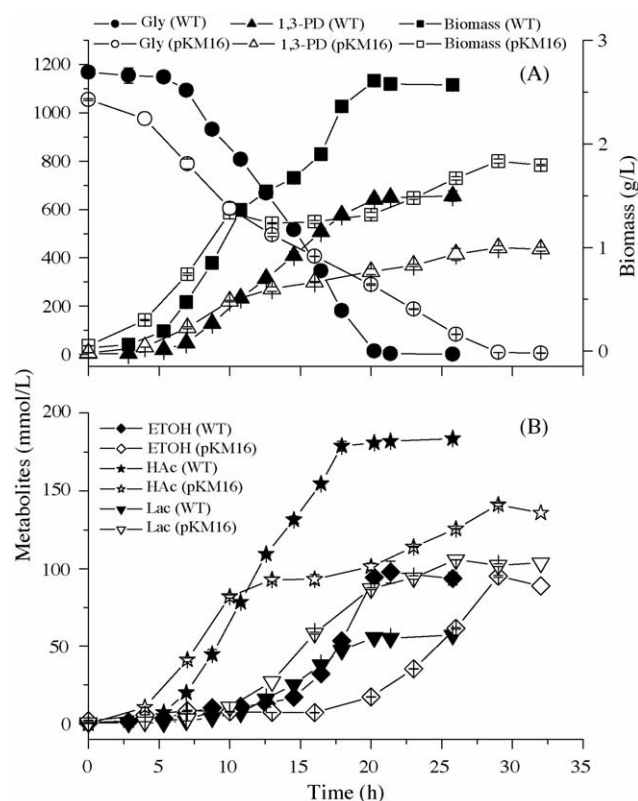


Fig. 6. Comparison of batch cultivations between the wild type *K. pneumoniae* DSM2026 and the recombinant DSM2026 (pKM16). The cultivation condition was the same as described in Fig. 4. (A) Glycerol consumption, growth and 1,3-propanediol production. (B) Products. Abbreviations are the same as those in Fig. 5 except Lac for lactate.

recombinant bearing the plasmid pKM16 and was about two fold higher than that of the wild type. The absolute amount of ethanol was not affected but with a delay of production for several hours in the recombinant (Fig. 6B). No apparent change was observed in the production of succinic acid and 2,3-butandiol (data not shown).

#### 4. Discussion

*K. pneumoniae* DSM2026 and the recombinant derivative strain DSM2026 (pKM13) exhibited efficient growth in batch culture experiments, although glycerol concentration was employed in remarkably high initial quantities (around 100 g/L). No obvious inhibition phenomena were observed, and in both instances significant quantities of 1,3-propanediol were produced.

The results shown in Fig. 3 and Table 3 suggested the loss of the plasmids pKM13 and pKM15 in anaerobic continuous cultivations and flask cultures of *K. pneumoniae* with glycerol as sole carbon source. Many factors can affect this process. Both plasmids pKM13 and pKM15 are descendants of the ColE1 plasmid pBR322. For example, a random distribution process of ColE1 plasmids into the daughter cells at cell division can yield plasmid-free daughter cells [24], leading to plasmid instability. Moreover, plasmid-bearing cells normally grow more slowly than plasmid-free cells because of an additional metabolic burden and therefore the plasmid-free cells become dominant in the culture. The situation is even worse in continuous cultures because of washout of the plasmid-bearing cells. However, the intrinsic random distribution of the pBR322-derived plasmids should not be the main reason for the plasmid loss. According to our experiences the plasmid pBR322 was quite stable in the studied continuous or batch cultivations under selective pressure. Furthermore, it was also found that the plasmid pTC53, a derivative of pBR322 bearing a synthetic operon of *dhaB* and *dhaT* genes under the control of an inducible *trc* promoter, is very stable in the continuous culture without induction. This fact excludes the possibility that the vector itself or the sequence of structural genes (*dhaB* or *dhaT*) causes the instability.

Another possible reason is due to the overexpression of heterogeneous proteins. It has been stated that the overexpression of plasmid-encoded protein can be a principal factor to impose a heavy metabolic burden on the host because a large amount of energy is consumed during protein biosynthesis [25]. The slow growth of the recombinant harbouring the plasmid pKM13 in batch cultivation seems to support this point. However, it cannot explain why pKM13 is more stable than pKM15 since the only difference between pKM13 and pKM15 is that pKM13 bears an additional *dhaT* gene the expression of which causes additional energy consumption and therefore a heavier metabolic burden, theoretically. To explain the instability of pKM15 and pKM13, the feature of genes harboured by the plasmids should be carefully considered.

The enzyme GDHt encoded by the *dhaB* genes is responsible for the dehydration of glycerol, yielding 3-HPA which is known as a natural antimicrobial agent [26]. It is suggested that the toxicity of 3-HPA could be the result of inhibition of DNA synthesis [27]. It has been postulated that the reactivity of the aldehyde group of 3-HPA causes DNA damage similarly to formaldehyde. Addition of 3-HPA at a final concentration of 30 mM to cultures of *K. pneumoniae*, *C. freundii* and *Enterobacter agglomerans* in the exponential growth phase caused an immediate cessation of the growth and glycerol consumption [28]. Up to 24 mM of 3-HPA could be accumulated during glycerol fermentation by native *K. pneumoniae* and its accumulation was related to the cessation of growth and the lower rates of substrate consumption and 1,3-propanediol formation [28]. The secreted 3-HPA was later assimilated and then the growth and product formation were recovered to some extent [29].

We postulate that the instability of plasmids bearing *dhaB* genes is associated with the toxicity of 3-HPA (Fig. 7). When glycerol is available, the overexpression of GDHt can lead to a rapid accumulation of the toxic intermediate 3-HPA which seriously damages the host cells. The damage can be so strong (and even irreversible) that the plasmid-bearing cells can no more grow or at least more slowly, dependent on how high the expression level of GDHt is. The cells that lose the plasmid then grow apparently faster, depending on how strong the selective

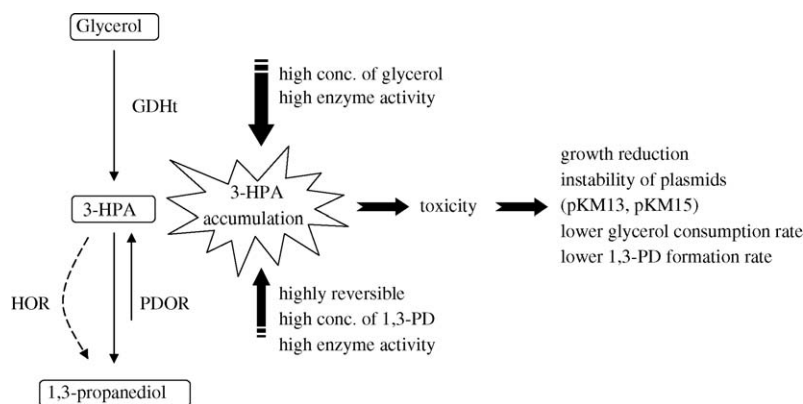


Fig. 7. Postulated accumulation of 3-HPA due to imbalanced activities of the enzymes glycerol dehydratase (GDHt) and 1,3-propanediol oxidoreductase (PDOR) and high reversibility of PDOR which further leads to cellular toxicity, plasmid instability and lower 1,3-propanediol formation rate in recombinant strains of *K. pneumoniae*.

pressure is. In our experimental studies, penicillin and carbenicillin were used as selective pressure. From the genomic sequence of a clinical isolation of *K. pneumoniae* strain MGH 78578 (The Genome Sequencing Center at Washington University Medical School, <http://genomeold.wustl.edu/>), one gene encoding a beta-lactamase was found (data not shown), indicating a potential high resistance of this organism to the antibiotics of penicillin family. We also observed that the cells of the studied strain DSM2026 can grow slowly on antibiotic agar plates when too many cells (>1000 cells per plate) are inoculated at the same time, showing that the strain is obviously resistant to penicillin to some extent. Furthermore, penicillin and carbenicillin can be degraded by cells expressing beta-lactamase, as in the case of cells bearing plasmids or even the wild type. Considering all these facts, it is not surprising that the wild type can outgrow the cells overexpressing GDHt. Therefore a complete or serious loss of plasmid was observed in our experiments, both in continuous and batch fermentations.

To verify this hypothesis, a determination of the extracellular concentration of 3-HPA in batch cultures of DSM2026 (pKM15) and DSM2026 (pBR322) was performed by using a colorimetric method [30]. Surprisingly, a similar low amount of 3-HPA was detected in the fermentation broth of the strain carrying plasmid pKM15 in comparison with the strain DSM2026 (pBR322). A measure of the plasmid stability showed that about 65% of the recombinant cells (strain pKM15) lost the plasmid in 30 min after the inoculation. And at this time point the biomass level was still very low (0.16 g/L). It is therefore reasonable that the amount of extracellular 3-HPA cannot reach a distinguishable level in such a short time by such low amount of cells. We also tried to measure the intracellular concentration of 3-HPA. However, due to the presence of the notorious extracellular polysaccharide in *K. pneumoniae* and thus the difficulty in fast centrifugation and cell disruption, the results remain unsatisfied till now.

Interestingly, the plasmid pKM13 harbouring both *dhaB* and *dhaT* is significantly more stable than pKM15 harbouring merely *dhaB*. The coordinated overexpression of PDOR in the recombinant pKM13 will drain the toxic intermediate 3-HPA to 1,3-PD in an accelerated way. Consequentially, the accumulation and toxicity of 3-HPA in the cells harbouring pKM13 should be lower than in the cells bearing pKM15, and therefore pKM13 is more stable than pKM15. This is consistent with our experimental observation (Fig. 3 and Table 3).

However, the excessive overexpression of *dhaT* alone is also not always favourable (Fig. 6), though the recombinant carrying the plasmid pKM16, exhibiting an increased expression of PDOR, showed a high plasmid stability (Table 3). The benefit to the cell growth and the 1,3-PD production was only shown in the first 10 h at the time 1,3-PD concentration is relatively low. During the middle stage of fermentation after 10 h, the growth of the pKM16 recombinant did not recover from the negative influence of 3-HPA as quickly as the wild type. The metabolic flux was channelled to the synthesis of lactate, instead of acetate as in the wild type, for almost another 10 h until the cells gained their ability to grow from 20 h (Fig. 6). The metabolic redirection to lactate is likely due to NADH excess. Lower GDHt activity in

the pKM16 recombinant (Fig. 4) caused slower production of 3-HPA and thus inadequate consumption of the reducing power NADH despite of the higher PDOR activity. Since NAD<sup>+</sup> is essential to keep the glycerol oxidative pathway active to generate ATP, the cells under the NADH excess (NAD<sup>+</sup> depletion) state might switch on other NAD<sup>+</sup> generation pathways (such as lactate synthesis pathway) and/or turn off some NADH generation reactions (such as the reaction catalysed by pyruvate dehydrogenase). Direct measurement of cellular NADH/NAD<sup>+</sup> ratio in the future would be helpful to verify this possibility. The carbon recovery was calculated as defined by Menzel [23] for batch culture considering the CO<sub>2</sub> release coupled with the production of final organic products and the fermentation volume change due to pH control. The carbon recovery of the pKM16 recombinant is 10% lower than that of the wild type. The reason for the carbon losses in the recombinant was not yet understood, probably because other unknown metabolites were produced but not determined in this study.

PDOR catalyzes a reversible reaction [31–33]. In *K. pneumoniae* the physiological forward reaction rate (from 3-HPA to 1,3-PD) is supposed to be four times of the reverse reaction [12]. However, the reverse reaction can become more and more significant with the accumulation of 1,3-PD in the fermentation broth, possibly leading to an accumulation of 3-HPA and toxicity to the cell. The enhanced expression of PDOR in the recombinant carrying the plasmid pKM16 may strengthen the reverse reaction. This could explain why the cells harbouring pKM16 could not recover from growth cessation in the later phase as the wild type. The inhibitory effect of 1,3-PD on cell growth was confirmed in *C. butyricum* CNCM1211 by addition of 1,3-PD to the initial fermentation medium [34]. The  $\mu_{\max}$  was inversely proportional to the initial concentration of 1,3-PD (0–65 g/L). 1,2-Propanediol also has a similar inhibitory effect. These findings can now be understood by considering the reversibility of PDOR and the subsequent toxicity caused by 3-HPA (Fig. 7). It is noted, though, that other strains (natural or mutants) present significantly increased tolerance to 1,3-PD found in the culture medium [35–37]. The performance of *C. butyricum* F2b is especially interesting since it showed significant growth without inhibition on high initial quantities of raw glycerol, a substrate containing several factors such as methanol, salts that inhibit microbial growth, and tolerated up to 80 g/L of 1,3-PD added into the medium in a continuous anaerobic fermentation [38–40]. In our recent proteomic study with the wild type strain of *K. pneumoniae* [29], a hypothetical oxidoreductase (HOR) was identified in the later fermentation phase. HOR was hypothesized to catalyze reactions from 3-HPA to 1,3-PD and replace the function of PDOR but with a much lower reversibility, meaning less accumulation of the toxic 3-HPA. The catalytic activity of HOR has been recently confirmed by Zhang et al. [41]. The specific activity of HOR under the induction of 1.0 mM IPTG in *E. coli* reached 120 U/mg protein in comparison to 0.5 U/mg protein without induction [41]. If the hypothesis of less ability of HOR catalysing the reverse reaction is true, it is HOR but not PDOR that should be overexpressed to accelerate the redraw of toxic 3-HPA to 1,3-PD formation.

## 5. Conclusion

The excessive expression of GDHt and/or PDOR was demonstrated to be not favourable for overproducing 1,3-PD in the glycerol fermentation by *K. pneumoniae*. It caused a serious toxicity to cell growth and instability of plasmids harbouring the corresponding genes, probably due to an imbalanced conversion of glycerol to the toxic intermediate 3-hydroxypropionaldehyde and its further conversion to 1,3-propanediol and the associated accumulation of 3-hydroxypropionaldehyde. Based on these results, it is concluded that any attempts for further metabolic engineering of 1,3-PD production in *K. pneumoniae* should carefully control the accumulation of 3-hydroxypropionaldehyde via a balanced expression of GDHt and PDOR. The effect of the *dhaR* overexpression was also investigated in *K. pneumoniae*. The resulted over-expression of PDOR confirmed the role of DhaR as a positive regulator of the *dhaT* gene.

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