J Ind Microbiol Biotechnol (2010) 37:707–716
DOI 10.1007/s10295-010-0714-9

Inactivation of *dhaD* and *dhaK* abolishes by-product accumulation during 1,3-propanediol production in *Klebsiella pneumoniae*

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Received: 4 December 2009 / Accepted: 18 March 2010 / Published online: 9 April 2010

Abstract 1,3-Propanediol (1,3-PD) can be used for the industrial synthesis of a variety of compounds, including polyesters, polyethers, and polyurethanes. 1,3-PD is generated from petrochemical and microbial sources. 1,3-Propanediol is a typical product of glycerol fermentation, while acetate, lactate, 2,3-butanediol, and ethanol also accumulate during the process. Substrate and product inhibition limit the final concentration of 1,3-propanediol in the fermentation broth. It is impossible to increase the yield of 1,3-propanediol by using the traditional whole-cell fermentation process. In this study, *dhaD* and *dhaK*, the genes for glycerol dehydrogenase and dihydroxyacetone kinase, respectively, were inactivated by homologous recombination in *Klebsiella pneumoniae*. The *dhaD*/*dhaK* double mutant (designated TC100), selected from 5,000 single or double cross homologous recombination mutants, was confirmed as a double cross by using polymerase chain reaction. Analysis of the cell-free supernatant with high-performance liquid chromatography revealed elimination of lactate and 2,3-butanediol, as well as ethanol accumulation in TC100, compared with the wild-type strain. Furthermore, 1,3-propanediol productivity was increased in the TC100 strain expressing glycerol dehydratase and 1,3-PDO dehydrogenase regulated by the arabinose P_{BAD} promoter. The genetic engineering and medium formulation approaches used here should aid in the separation of 1,3-propanediol from lactate, 2,3-butanediol, and ethanol and lead to increased production of 1,3-propanediol in *Klebsiella pneumoniae*.

Keywords 1,3-Propanediol · *dhaD*/*dhaK* · *Klebsiella pneumoniae* · Gene expression · Gene knockout

Yu-Tze Horng and Kai-Chih Chang contributed equally to this work

Electronic supplementary material The online version of this article (doi:10.1007/s10295-010-0714-9) contains supplementary material, which is available to authorized users.

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Introduction

1,3-Propanediol (1,3-PD) is a valuable chemical product, with particular use as a suitable monomer for polycondensation to produce polyesters, polyethers, and polyurethanes [33]. Polymers generated by 1,3-propanediol have several advantageous characteristics, such as good light stability, biodegradability, and elasticity. To date, 1,3-PD has been produced using two methods, chemical synthesis and microbial conversion. Biosynthesis is a particularly attractive option because it typically uses renewable feedstock and does not generate toxic by-products.

Glycerol is converted to 1,3-propanediol by several bacteria, including strains of the genera *Klebsiella*, *Citrobacter*, and *Clostridium* [5, 12]. Genes encoding the functionally linked activities of glycerol dehydratase (*dhaB*), 1,3-PDO dehydrogenase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*)

are encompassed by the *dha* regulon in *Klebsiella pneumoniae* [11, 36]. In pathways involving anaerobic metabolism of glycerol [2], glycerol is oxidized by an NAD-linked glycerol dehydrogenase (DhaD, GDH, EC 1.1.1.6) to dihydroxyacetone (DHA), phosphorylated by DhaK (oxidative branch), and then funneled to the central metabolism, or is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by coenzyme B12-dependent glycerol dehydratase (reductive branch). Glycerol dehydratase (GDHt) consists of three different subunits encoded by three genes, one of which is *dhaB*. 3-Hydroxypropionaldehyde is subsequently reduced to 1,3-propanediol by the NADH-linked 1,3-propanediol oxidoreductase (DhaT, PDOR, EC 1.1.1.202) [2]. However, during the biological production of 1,3-propanediol, a series of by-products (such as acetic acid, lactate, 2,3-butanediol, and ethanol) increase the purification cost and time. In particular, the similar boiling points of 2,3-butanediol and 1,3-propanediol can cause purification difficulties [32]. One solution is the application of purified key enzymes. The key enzymes of glycerol fermentation include GDH in the oxidative branch, and GDHt and PDOR in the reductive branch [6].

Here, we describe a genetic engineering strategy to decrease by-product production in *K. pneumoniae*. A mutant strain deficient in GDH (DhaK) activity was generated by insertion-deletion of a streptomycin resistance marker into the *dhaD–dhaK* genes. This mutant strain was unable to produce by-products such as lactate, 2,3-butanediol, and ethanol. 1,3-Propanediol production by the mutant expressing enzymes in the reductive branch, namely DhaB and DhaT, was also increased. Further, we optimized the medium for growth of the mutant strain to increase 1,3-propanediol production.

Materials and methods

Bacterial strains, media, and growth conditions

The bacterial strains and plasmids used in this study are presented in Table 1. LB was used as a rich medium for the routine growth of *K. pneumoniae* strains. The composition of the culture medium for *K. pneumoniae* was the same as used in previous reports [18, 19]. The glycerol concentration in the medium was 20 g l^{-1}. Shake flask microaerobic cultivation at 200 rpm and 37°C was used for seed culture, initial investigation of GDHt and PDOR overexpression using different plasmids, and study of plasmid stability in variant conditions. Microaerobic batch cultivation (0.5 vvm, 200 rpm) was carried out in a 5L bioreactor (FIRSTEC, Taiwan) with a working volume of 1 l. The initial glycerol concentration in the medium was 20 g l^{-1}. pH was maintained in the range of 6.8–7.4.

Enzymes and chemicals

DNA restriction and modification enzymes were purchased from Roche (Mannheim, Germany). *Taq* polymerase and PCR-related products were obtained from Perkin-Elmer (Boston, MA) or Takara Biomedicals (Shiga, Japan). Other laboratory-grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Merck (Schwabach, Germany).

Recombinant DNA techniques

Unless otherwise indicated, standard protocols were used for DNA/DNA hybridization, plasmid and chromosomal DNA preparation, transformation, electroporation, PCR, restriction digestion, agarose gel electrophoresis, DNA recovery from agarose gels, ligation, and conjugation [28]. PCR DNA amplicons were cloned by using pCR 2.1 and the TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequencing and analysis were performed by using a Perkin-Elmer Autosequencer model 377 with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster, CA). The DNA sequences of PCR products were confirmed by sequencing both strands from two or three independent reactions.

Preparation of cell extracts

Crude cell extracts were prepared by sonication of cell paste and subsequent centrifugation. Cell paste was obtained by centrifugation of fermentation broth at 7,000 g for 10 min at 4°C. The paste was washed in 20 mmol l^{-1} Tris–HCl buffer (pH 8.0) or 50 mol l^{-1} potassium phosphate buffer (pH 8.0), centrifuged as described above, and resuspended in a small amount of the appropriate assay buffer. Cells were disrupted by sonication for 5 min on ice at a duty cycle of 60% with 1-s cycles. Cell debris was removed by centrifugation at 14,000*g* for 10 min at 4°C in a microcentrifuge.

High-performance liquid chromatography analysis of metabolites

Metabolites present in fermentation broth were analyzed by using an Hitachi high-performance liquid chromatography system with a refractive index detector and ICE-87H3 organic acids column at a flow rate of 0.6 ml min^{-1} and column temperature of 65°C. The mobile phase was 5 mmol l^{-1} H_2SO_4. Samples were microfiltered through 0.45-μm membranes, prior to analysis.

Detection of glycerol dehydratase activity

The enzyme assay was described by Ahrens et al. [2]. Briefly, 1,2-propanediol was used as the substrate for the

Table 1 Bacterial strains, plasmids, and primers used in this study

Strains, plasmids, and primers	Characteristics	Source or reference
Strains		
Klebsiella pneumoniae	Clinical isolated strain	From NTUH
E.coli DH5α	F^-, Φ80d*lacZΔ*M15, (*lacZYA-argF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17*(r_k^-,m_k^+), *phoA*, *supE44*,λ^-, thi-1, *gyr*A96, *rel*A1	TaKaRa Bio
E.coli S17-1	λ^-*-pir* lysogen of S17-1 [*thi pro hsdR hsdM*$^+$ *recA* RP4 2-Tc::Mu-Km::Tn*7* (Tp^r Sm^r)]; permissive host able to transfer suicide plasmids requiring the Pir protein by conjugation to recipient cells	[28]
TC100	*Klebsiella pneumoniae* Δ*dhaD,* Δ*dhaK*, Sm^r	This study
TC101	TC100 transform with pBAD24 or pBAD33 vector only	This study
TC102	TC100/pTC2, Cm^r	This study
TC103	TC100/pTC3, Km^r	This study
TC104	TC100/pTC2, pTC3, Km^r, Cm^r	This study
Plasmids		
pBAD33	pACYC184 ori, arabinose regulation, Cm^r	[12]
pBAD18-Kan	pBR322 ori, arabinose regulation, Km^r	[12]
pTC-1	pUT-sm^r::*dhaD'*:: Ω cassette::*dhaK'*::Km, Sm^r, Km^r	This study
pTC-2	pBAD33:: *dhaB*, Cm^r	This study
pTC-3	pBAD18-Kan:: *dhaT*, Km^r	This study
Primers		
dhaD-FF	5′-GTCGACGGCGGACTCATCAAGAATAAAC-3′	This study
dhaD-FR	5′-AAGCTTGCGGAAGGGGAAACCATC-3′	This study
dhaK-BF	5′-AAGCTTATGACGAGACGCTGACGC-3′	This study
dhaK-BR	5′-GAATTCTCCCCTGGCTCCGCTTTG-3′	This study
dhaB-F	5′-CAGCTGCCTTTTGAGCCGATGAAC-3′	This study
dhaB-R	5′-AAGCTTAGATCTATCAATCCCGGCTATTAAC-3′	This study
dhaT-F	5′-CCCGGGAGATCTCCTGAAGCGAGAAGGTATATTATG-3′	This study
dhaT-R	5′-GTCGACAGATCTGCCCCCTCGTTAACAGTC-3′	This study

GDHt assay. The reaction was performed under strict exclusion of light. The assay mixture contained 0.05 mol l^{-1} KCl, 0.2 mol l^{-1} 1,2-propanediol, 15 mmol l^{-1} coenzyme B12, and 0.035 mol l^{-1} potassium phosphate buffer solution in a total volume of 1 ml (pH 7.0). The assay was started by the incubation with cell extracts at 37°C. After 10-min incubation, the reaction was terminated by adding 1 ml of 0.1 mol l^{-1} potassium citrate buffer (pH 3.6). For developing color after the addition of 0.5 ml of 0.1% MBTH (3-methyl-2-benzothiazolinone-hydrazone) solution, the mixture was re-incubated for 15 min at 37°C. The color formed was detected at 305 nm after the addition of 1 ml of distilled water. For all samples, repeated determinations were performed with different concentrations.

Detection of 1,3-propanediol oxidoreductase activity

The activity of 1,3-propanediol oxidoreductase (EC 1.1.1.202) is usually assayed by using the reverse rather than the physiological reaction due to the instability of 3-HPA, which is also commercially unavailable [1, 10, 13]. According to Forage and Foster [10], the reaction mixture contained 30 mmol l^{-1} ammonium sulfate, 0.1 mol l^{-1} 1,3-propanediol, 2 mmol l^{-1} NAD (adjusted to pH 7.0 with 1 mol l^{-1} NaOH), and 0.1 mol l^{-1} potassium carbonate buffer solution in a volume of 1 ml (pH 9.0). The reaction was started by adding cell extract, and increase in $NADH_2$ was monitored photometrically at 340 nm with a spectrophotometer.

Results

Construction of *dhaD–dhaK* gene-deleted mutant *K. pneumoniae*

For construction of the *dhaD* and *dhaK* gene-deleted mutant, a 2-kb streptomycin (Sm)-resistant Ω cassette excised from pHP45Ω was specifically inserted into the *dhaD–dhaK* gene sequence in *K. pneumoniae* [22, 28]. Briefly, the 3′ regions of *dhaD* and *dhaK* genes were

amplified by polymerase chain reaction (PCR) by using the primer pairs dhaD-FF/dhaD-FR and dhaK-BF/dhaK-BR, respectively. The two amplified products were TA cloned into pCR 2.1 (Invitrogen), followed by excision of the *Sal*I/*Hind*III and *Hind*III/*Eco*RI fragments, respectively. The two DNA fragments, together with the Ω cassette, were ligated into the *Sal*I/*Eco*RI-digested pUTmini-Tn*5*-Km1 suicide vector [8] to form pUT-dhaD′::Sm^r::dhaK′, designated pTC-1. For gene inactivation by homologous recombination, pTC-1 was transferred from *Escherichia coli* S17-1(λ pir) to *K. pneumoniae* by conjugation [15]. Transconjugants were spread on LB plates containing streptomycin (50 μg ml^{-1}) and tetracycline (13 μg ml^{-1}). Mutant candidates were screened and confirmed by colony PCR by using the primer pair dhaD-FF/dhaK-BR (Fig. 1). The resultant *dhaD–dhaK* gene-deleted mutant strain was denoted *K. pneumoniae* TC100.

The mutant strain, TC100, loses the function to produce by-products during 1,3-propanediol production

The two genes *dhaD* and *dhaK* encode glycerol dehydrogenase and dihydroxyacetone kinase, respectively. These enzymes are involved in glycerol metabolism leading to production of pyruvate, which subsequently forms metabolites such as lactate, acetate, 2,3-butanediol, and ethanol in *K. pneumoniae* [36]. To determine the function of glycerol metabolism in *K. pneumoniae* TC100, the bacterial strain was cultured in medium containing glycerol as a carbon source, and metabolic products were analyzed by using high-performance liquid chromatography (HPLC) after a 24-h incubation period at 37°C. HPLC results showed that, compared with the wild-type strain, the TC100 mutant could not produce lactate (retention time of 13.08 min), 2,3-butanediol (19.07 min), or ethanol (22.64 min; Fig. 2 and Supplementary data). Analysis of physiological changes confirmed successful generation of mutant TC100. Our findings support the potential benefits of decreased by-product synthesis during microbial production of 1,3-propanediol by using TC100 in medium containing glycerol as a carbon source.

Yield of 1,3-propanediol is enhanced in the mutant strain TC100

In *Klebsiella,* glycerol is metabolized either oxidatively or reductively [36]. In the oxidative pathway, the NAD$^+$-dependent glycerol dehydrogenase catalyzes the conversion of glycerol to dihydroxyacetone, which is processed by glycolysis [7, 16, 17]. The reductive pathway is catalyzed by coenzyme B_{12}-dependent glycerol dehydratase and related diol dehydratases [14], which convert glycerol to 3-hydroxypropionaldehyde [24, 31], and the NADH + H$^+$-dependent enzyme 1,

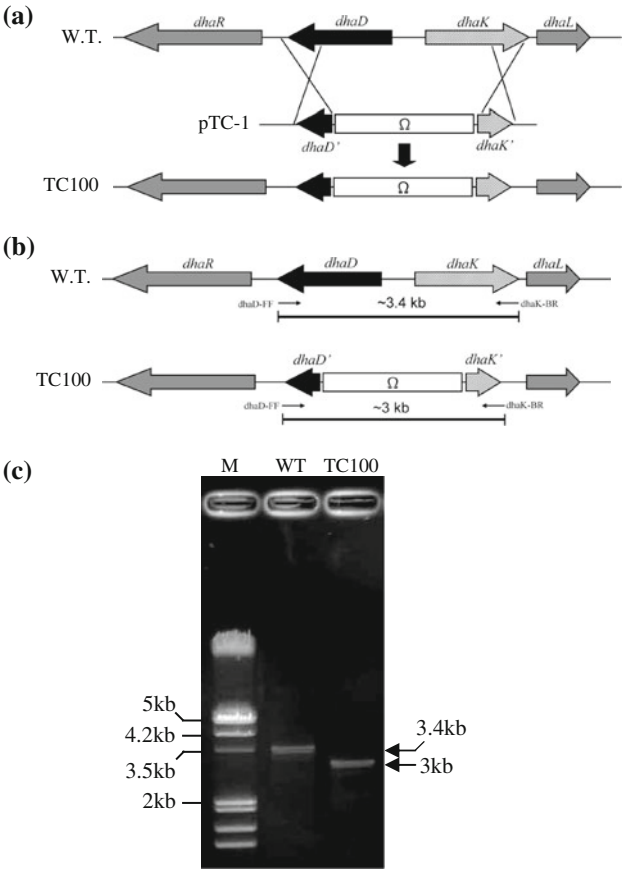

Fig. 1 Genetic map of the *K. pneumoniae dhaD–dhaK* gene locus and construction of the *dhaD–dhaK* deletion mutant in *K. pneumoniae*, TC100. **a** Construction of *dhaD–dhaK* gene knockout mutant strain, TC100, by insertion of a streptomycin-resistance Ω cassette into *K. pneumoniae dhaD-dhaK* genes through homologous recombination. pTC-1 is a recombinant suicide vector for insertion-deletion mutagenesis of *dhaD–dhaK* genes. **b** Physical map of wild-type and mutant TC100. **c** Genetic confirmation of the insertion-deletion mutant strain using PCR. *M* marker, *WT* wild-type, *TC100 K. pneumoniae dhaD–dhaK* gene deletion mutant strain

3-propanediol dehydrogenase, which reduces 3-hydroxypropionaldehyde to 1,3-propanediol and regenerates NAD$^+$ [2, 21].

We hypothesized that 1,3-propanediol production is increased by enhancing metabolic flux to the reducing pathway and blocking the oxidative pathway (Fig. 3). Our HPLC results show that extracellular metabolites from the oxidative pathway, specifically, lactate, 2,3-butanediol, and ethanol, are absent from the mutant TC100 strain (Fig. 2). The 1,3-propanediol concentration was also analyzed. The specific 1,3-propanediol productivity of individual cells was slightly higher in the TC100 mutant strain than the wild-type strain (Table 2). Each cell yield of 1,3-propanediol by TC100 was increased by about 2.06-fold relative to the wild-type strain after 48-h incubation (Table 2). Our results indicate that TC100 metabolically converts glycerol into 1,3-propanediol more efficiently, but produces less biomass and

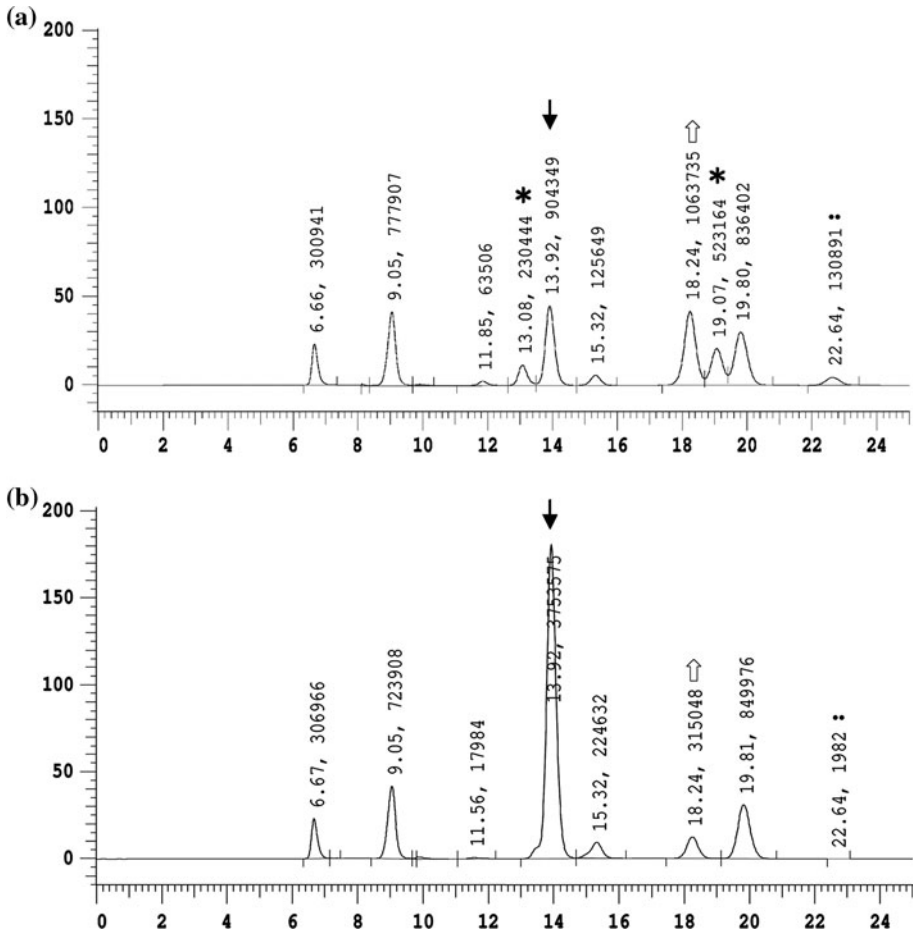

Fig. 2 Measurement of culture medium components by HPLC. Wild-type (**a**) and TC100 mutant (**b**) strains were analyzed using the 5 mmol l^{-1} H_2SO_4 as mobile phase. Peaks at retention times of 13.08 min (lactate) and 19.07 min (2,3-butanediol) were present in the wild-type strain (*asterisks*), but absent in TC100. The peak at a retention time of 22.64 min (ethanol) was decreased in TC100 (··). Glycerol, retention time of 13.92 min, was consumed more by the wild-type than mutant strain (*down arrow*). Peak at a retention time of 18.24 min is due to1,3-propanediol (*up arrow*)

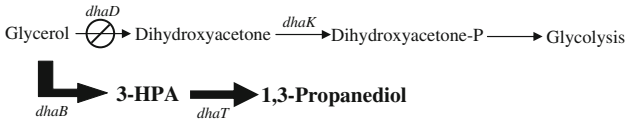

Fig. 3 Engineering the glycerol metabolic pathway. *dhaD* glycerol dehydrogenase, *dhaK* dihydroxyacetone kinase, *3-HPA* 3-hydroxypropionaldehyde

total 1,3-propanediol, compared with the parent strain. We further improved biomass and total productivity of 1,3-propanediol in the mutant strain by genetic modification and adjusting the incubation conditions.

1,3-Propanediol production is increased with *dhaB* or *dhaT* trans-overexpression in TC100

In *K. pneumoniae*, 1,3-propanediol 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 reaction of glycerol to 3-hydroxypropionaldehyde (3-HPA) [26, 30]. Subsequently, 3-HPA is reduced to 1,3-propanediol by 1,3-propanediol oxidoreductase (PDOR), which is encoded by the gene *dhaT* under control of the reducing factor $NADH_2$ [9, 29]. The GDHt and PDOR enzymes obtained from *K. pneumoniae* have been overexpressed in *E. coli* [27, 31] to produce 1,3-propanediol in a fed-batch fermentation process, using glucose and glycerol as co-substrates. Moreover, increasing the dehydratase activity in the native producer *K. pneumoniae*, may increase the level of 1,3-propanediol fermentation [35].

To increase 1,3-propanediol synthesis, *dhaB* and *dhaT* were trans-overexpressed in *K. pneumoniae* TC100. Plasmids pTC2 and pTC3 carrying *dhaB* and *dhaT*, respectively, under the control of the arabinose P_{BAD} promoter, were constructed (Table 1) and transformed into *K. pneumoniae* TC100 by electroporation. Recombinant bacteria were grown in shaker flasks with glycerol as the sole carbon source and 0.1% arabinose as an inducer, followed by detection of enzyme activities (Table 3). The specific activities of GDHt in the TC102 strain and PDOR in the TC103 strain were approximately 6- and 6.5-fold higher than those in the TC101 control strain, respectively.

Further quantitative experiments were performed to confirm the effects of *dhaB* and/or *dhaT* overexpression on

Table 2 The flask culture comparison between mutant strain TC100 and wild-type strain

Strain/factors	TC100		Wild-type		Ratio of TC100 to wild-type (%)	
Flask culture time	24 h	48 h	24 h	48 h	24 h	48 h
Bacteria number ($\times 10^8$)	0.77	0.69	1.45	1.35	53.10	51.11
Glycerol consumed (g l^{-1})	8.07	8.97	13.57	17.96	59.47	49.94
1,3-PD (g l^{-1})	1.82	1.92	3.11	3.67	58.52	52.32
Ethanol (g l^{-1})	0	0	0.67	1.48	0	0
Acetic acid (g l^{-1})	1.51	1.92	0.85	0.69	177.12	277.62
Lactic acid (g l^{-1})	0	0	1.01	1.34	0	0
2,3-Butanediol (g l^{-1})	0	0	1.74	3.38	0	0
Specific 1,3-PD producing capability of each cell (g)	2.36	2.78	2.14	2.72	110.28	102.20
1,3-PD productivity of each cell (g $l^{-1}h^{-1}$)	0.0983	0.0579	0.0892	0.0567	110.20	102.21
1,3-PD yield of each cell	0.29	0.31	0.16	0.15	181.25	206.67

Culture conditions: bacteria cells were cultured in 50-ml flasks and incubated at 37°C

1,3-propanediol production. Each strain was cultured in flasks containing culture medium in which arabinose was added to a concentration of 0.1% to induce *dhaB* or *dhaT* gene expression after 12-h incubation. The 1,3-propanediol concentration of each strain was analyzed after 24 h of incubation. The 1,3-propanediol concentrations were increased by approximately 1.3-fold in TC102 and approximately 2.1-fold in TC103, compared with the vector-only strain TC101. Additionally, 1,3-propanediol production was increased more than 2.4-fold in the *dhaB* and *dhaT* co-transformant strain TC104 compared with TC101 (Table 3). These results collectively indicate that overexpression of DhaB and DhaT increases 1,3-propanediol production in *K. pneumoniae* TC100.

Increased biomass and 1,3-propanediol production by pH adjustment in the medium

Although some by-products were eliminated during the production of 1,3-propanediol with the mutant strain TC100, using glycerol as carbon source, about 2.8-fold increased acetate accumulation was observed, compared with the wild-type strain (Table 2). High concentrations of acetate affect cell growth and decrease enzyme activities in bacterial cells [23]. The pH value of the culture medium was reduced to 4.76 in the TC100 mutant strain after 24 h of incubation (Table 4). Simultaneously, the growth rate of the mutant TC100 strain slowed relative to the wild-type strain. The bacterial cell count of TC100 incubated for 24 h

Table 3 Overexpression of glycerol dehydratase (GDHt) and/or 1,3-propanediol oxidoreductase (PDOR) in each recombinant strain

Strain/plasmid	Genes on plasmid	GDHt (*dhaB*; U mg^{-1} protein)	PDOR (*dhaT*; U mg^{-1} protein)	1,3-PD conc. (g l^{-1})/glycerol consumed	pH adjusted 1,3-PD conc. (g l^{-1})/glycerol consumed
Mutant overexpression strains					
TC101	Vector only	0.0112	0.0195	1.57/7.14	3.13/14.65
TC102	*dhaB*	0.0682	0.0187	1.99/8.29	3.26/15.82
TC103	*dhaT*	0.0132	0.1259	3.23/13.46	4.16/16.12
TC104	*dhaB* and *dhaT*	0.0612	0.1195	3.82/15.28	4.68/17.95
Wild type overexpression strains					
WT/vector	Vector only	0.0392	0.0263	3.43/14.29	ND
WT/pTC2	*dhaB*	0.1036	0.0256	3.88/15.52	ND
WT/pTC3	*dhaT*	0.0412	0.1623	4.84/19.29	ND
WT/pTC2, pTC3	*dhaB* and *dhaT*	0.1089	0.1572	5.68/19.25	ND

Culture conditions: bacteria cells were cultured in 50-ml flasks and incubated at 37°C. *ND* not detected

Table 4 Comparison of 1,3-propanediol concentration with or without pH adjusted

Strain/factors	TC100		TC100 pH adjusted		Ratio of pH to TC100 (%)	
Flask culture time	24 h	48 h	24 h	48 h	24 h	48 h
Bacteria number ($\times 10^8$)	2.45	2.93	4.16	4.51	169.8	153.9
pH value	4.76	4.72	6.35	7.55	133.4	160.0
Glycerol consumed (g l^{-1})	11.99	12.11	19.86	20.00	165.6	165.2
1,3-PD (g l^{-1})	1.65	1.72	4.05	3.77	245.5	219.2
Specific 1,3-PD producing capability of each cell (g)	0.67	0.59	0.97	0.84	144.8	142.3

Culture conditions: bacteria cells were cultured in 1-l flasks and incubated at 37°C. The pH value was adjusted after 6- and 12-h incubation

in 50-ml flask cultures was almost half that of the wild-type strain (Table 2). We propose that improvement of the growth rate leads to increased 1,3-propanediol production in the TC100 strain. To increase the growth rate, we adjusted the pH value of culture medium to neutrality (pH 7.0) by adding sodium hydroxide (NaOH). Notably, the growth rate of TC100 was restored following pH adjustment. The bacterial cell count of TC100 in NaOH-treated medium was 1.69-fold higher than that cultured in untreated medium after 24-h incubation (Table 4). In view of the improvement in bacterial growth rates, the individual cell concentrations of 1,3-propanediol were further assessed. Compared with the strain cultured in non-adjusted pH medium, individual cells treated with NaOH for 24 h produced approximately 1.45-fold more 1,3-propanediol (Table 4). Total 1,3-propanediol production was increased by 2.45-fold after pH adjustment and incubation for 24 h, compared with the level in strains cultured in non-adjusted pH medium. The effect of pH value adjustment on 1,3-propanediol production form TC102, TC103, and TC104 were similar to the TC100 (Table 3). The concentrations of 1,3-propanediol were all enhanced in the neutralized medium. These results indicate that mutation of the *dhaD* and *dhaK* locus results in acetate accumulation, leading to low pH in the stationary phase. Adjustment of the medium pH to neutrality improves the growth rate and 1,3-propanediol production in the mutant TC100 strain. We further assessed the effect of pH control on the 1,3-propanediol production more precisely. The TC100 was grown in the 5L microaerobic bioreactor with 1-l working volume in which the pH was maintained in the range of pH 6.8–7.4. The concentration of 1,3-propanediol reached 7.7 g l^{-1} after 30-h incubation (Fig. 4) and is higher than that achieved by flask culture. In order to test whether the combination of genetic engineering and medium formulation is a useful tool, we evaluated the production of 1,3-propanediol by the mutant containing overexpressed *dhaB* or/and *dhaT* incubated in the neutralized medium. Compared with wild-type strain in the original medium, the concentration of 1,3-propanediol from TC104 in the pH-adjusted medium was increased 1.36-fold (Table 3).

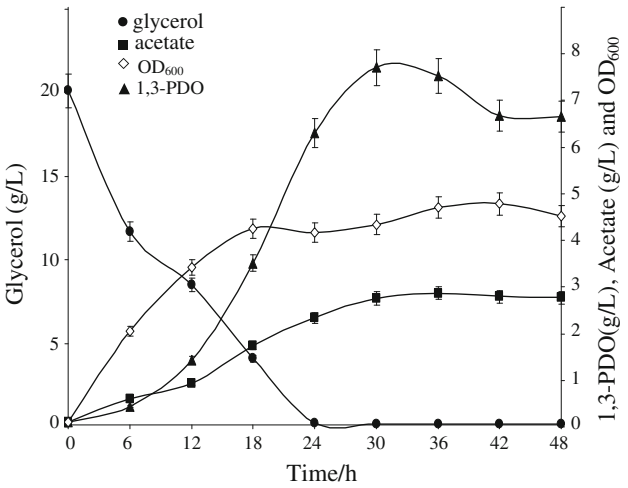

Fig. 4 Batch fermentation of *K. pneumoniae* TC100 with pH adjustment using flask culture conditions. *Circles* glycerol, *squares* acetate, *diamonds* optical density, *triangles* 1,3-propanediol

Simultaneously, the by-products lactate, 2,3-butanediol, and ethanol were not detected in TC104 incubated in the pH-adjusted medium after 24-h incubation. Together these results, *K. pneumoniae* deficient in *dhaD* and *dhaK* as well as overexpressing GDHt and PDOR in the medium with optimal pH value not only enhances the production of 1,3-propanediol but also reduces the by-products.

Discussion

In *K. pneumoniae*, glycerol is metabolized oxidatively or reductively. In the reductive process, glycerol is converted to 1,3-propanediol with relatively high yield and productivity [34]. We selected glycerol as a substrate because it is synthesized as a by-product during biodiesel formation, which involves in a chemical reaction of alcohol and vegetable or animal oils, fats, or grease. Based on the glycerol metabolic flux, we hypothesize that blockade of the oxidative pathway promotes the reductive pathway. In this study, we constructed a *dhaD–dhaK* gene-deleted mutant strain, TC100. This mutant strain had lost the

ability to generate by-products, including lactate, 2,3-butanediol, and ethanol, in the oxidative pathway (Fig. 2). The concentration of 1,3-propanediol produced by individual cells of the TC100 strain was similar to that produced by the wild-type strain. However, the yield of 1,3-propanediol was increased by about 2.06-fold in TC100, compared with the wild-type strain (Table 2). Yield is defined as the ratio of 1,3-propanediol generated relative to the amount of glycerol consumed. Our results indicate that the mutant strain TC100 converts glycerol to 1,3-propanediol more efficiently than the wild-type strain.

In previous investigations, recombinant *E. coli* over-expressing GDHt and PDOR enzymes from *K. pneumoniae* produced 1,3-propanediol [35]. Here, we expressed *dhaB* (encoding GDHt) and *dhaT* (encoding PDOR) driven by the P_{BAD} promoter in the mutant TC100 strain. The 1,3-propanediol concentration was increased in the recombinant *K. pneumoniae* mutant strain TC100 transformed with plasmids harboring *dhaB* or/and *dhaT* (Table 3). GDHt and PDOR activities were increased by 6.09- and 6.46-fold in TC102 and TC103, respectively, compared with TC101. It seems that the arabinose induction efficiencies of pTC2 and pTC3 are similar, leading to corresponding increases in enzymatic activity. However, the 1,3-propanediol concentration was increased more in TC103 than in TC102. One possible reason is that the product of GDHt (DhaB), 3-hydroxypropionaldehyde (3-HPA), is toxic for bacterial cells at high levels [3]. A combination of pTC2 (P_{BAD}::*dhaB*) and pTC3 (P_{BAD}::*dhaT*) in the recombinant *K. pneumoniae* mutant strain TC100 (TC104) generated higher levels of 1,3-propanediol compared with the wild-type strain; although the level was not significantly greater than that of TC103. It is possible that double arabinose P_{BAD} promoters (P_{BAD}::*dhaB* and P_{BAD}::*dhaT*) share the inducer (arabinose) or that 3-HPA is metabolized to 3-hydroxypropionic acid (3-HP) by aldehyde dehydrogenase (*aldH*) [20]. The enzyme activities of GDHt and PDOR are decreased in TC101, compared with wild-type (Table 3). Perhaps lower enzyme activities are due to the possibilities that *dha* regulon is regulated by transcription regulator DhaR or enzymes are affected in the lower pH condition. However, the *dhaB* and *dhaT* were under-controlled by arabinose P_{BAD} promoter, not by the native promoter in this study. The enzyme activities and effects of GDHt and/or PDOR are significantly increased in both wild-type strain and mutant strain in this strategy (Table 3).

Seo and colleagues reported elimination of by-product formation during 1,3-propanediol production in *K. pneumoniae* upon inactivation of the glycerol oxidative pathway [25]. These researchers constructed mutant strains defective in several genes; specifically, one strain was defective in *orfY, dhaT, orfW, orfX, dhaR, dhaD*, and *dhaK*, and the other in *orfY, dhaT, orfW, orfX*, and *dhaR*. The investigators complemented *dhaT, orfW*, and *orfX* genes in the mutants to identify the metabolites in the glycerol oxidative pathway that were not produced. They concluded that mutation of specific genes in the glycerol oxidative pathway, such as *dhaD* and *dhaK*, induces physiological changes in *K. pneumoniae*. However, chromosomal complementation is necessary to validate this assumption because multiple copies of plasmid complementation lead to gene overexpression. We disrupted the *dhaD* and *dhaK* genes in this study. Growth in glycerol medium and 1,3-propanediol production were lower in the mutant strain than in the parent strain, consistent with the previous findings of Seo et al. [25]; however, the pH of the medium was considerably lower in mutant strain cultures. The bacterial growth rate and 1,3-propanediol production of the mutant strain were restored to wild-type levels after neutralization of the medium pH. The by-products of the glycerol oxidative pathway (lactate, 2,3-butanediol, and ethanol) were still absent when the mutant strain was cultured in neutralized medium after 24-h incubation.

The disruption of *dhaD* and *dhaK* resulted in the absence of lactate, 2,3-butanediol, and ethanol in the mutant strain TC100, but produced more acetate than the wild-type strain. According to the biochemical pathways of glycerol fermentation, yields of 1,3-propanediol from the reductive branch pathway of glycerol consumed reducing power (NADH) which is produced from the oxidative branch pathway [4]. The carbon flux from the oxidation of glycerol leads to formation of pyruvate resulting in the production of the side-products lactate and 2,3-butanediol. The pyruvate is also oxidized to acetyl-CoA which leads to synthesis of acetate and ethanol. Other pathways exist, e.g., the acetyl-CoA could be available from oxidation of fatty acids and catabolism of amino acids in the case of deficiency of pyruvate. The yields of lactate, 2,3-butanediol, and ethanol compete with the biosynthesis of 1,3-propanediol for NADH while formation of acetate from acetyl-CoA generates ATP. In this study, we speculate that deficiency of *dhaD* and *dhaK* (in the oxidative branch pathway) directly blocks the formation of lactate and 2,3-butanediol and decreases the amount of NADH leading to low yields of ethanol. Simultaneously, it is suggested that the formation of acetate is enhanced for more ATP production to compensate the decreased amount of NADH (Table 2).

In conclusion, we have developed a biomethod involving in a combination of genetic engineering and medium formulation that can be used to eliminate by-products and increase 1,3-propanediol production. The *dhaD*/*dhaK*-deleted mutant harboring *dhaB* and *dhaT* (TC104) was cultured in glycerol medium with pH adjustment by adding NaOH. During the standard separation and purification of 1,3-propanediol, it is necessary to eliminate other contaminant by-products. The equipment designed for removal of

these by-products involves high costs. Our novel biomethod should reduce the costs of industrial fermentation and increase production of 1,3-propanediol by *K. pneumoniae*.

Acknowledgments This work was supported by grants from the National Science Council (NSC-97-2320-B-320-012-MY3) which were really appreciated.

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