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Inactivation of aldehyde dehydrogenase: A key factor for engineering 1,3-propanediol production by *Klebsiella pneumoniae*

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Abstract

Production of 1,3-propanediol (1,3-PD) from glycerol by *Klebsiella pneumoniae* is restrained by ethanol formation. The first step in the formation of ethanol from acetyl-CoA is catalyzed by aldehyde dehydrogenase (ALDH), an enzyme that competes with 1,3-PD oxidoreductase for the cofactor NADH. This study aimed to improve the production of 1,3-PD by engineering the ethanol formation pathway. An inactivation mutation of the *aldA* gene encoding ALDH in *K. pneumoniae* YMU2 was generated by insertion of a tetracycline resistance marker. Inactivation of ALDH resulted in a nearly abolished ethanol formation but a significantly improved 1,3-PD production. Metabolic flux analysis revealed that a pronounced redistribution of intracellular metabolic flux occurred. The final titer, the productivity of 1,3-PD and the yield of 1,3-PD relative to glycerol of the mutant strain reached 927.6 mmol L^{-1}, 14.05 mmol L^{-1} h^{-1} and 0.699 mol mol^{-1}, respectively, which were much higher than those of the parent strain. In addition, the specific 1,3-PD-producing capability (1,3-PD produced per gram of cells) of the mutant strain was 2-fold that of the parent strain due to a lower growth yield of the mutant. By increasing NADH availability, this study demonstrates an important metabolic engineering approach to improve the efficiency of oxidoreduction-coupled bioprocesses.

Keywords: Aldehyde dehydrogenase; *Klebsiella pneumoniae*; Inactivation; 1,3-Propanediol; Ethanol

1. Introduction

Biorefining processes of many chemicals, such as lactic acid (Datta, 1995; Davidson et al., 1995), L-phenylalanine (Chao et al., 2000; Takac et al., 1995) and mannitol (Wisselink et al., 2002), have shown significant advantages over the conventional chemical processes. Many bioconversion processes are coupled with oxidoreductive reactions, which offer considerable potentials for the production of commodity or fine chemicals, especially for those requiring stereospecificity (Berrios-Rivera et al., 2002). However, the complexity of metabolism normally leads to the diversity of metabolic products, which usually decreases the yield of the target product and increases the cost of downstream separation.

Recently, biosynthesis of 1,3-propanediol (1,3-PD) has attracted worldwide interests (Menzel et al., 1997a, b; Shelley and D'Aquino, 1999). *Klebsiella pneumoniae* and *Clostridium butyricum* can convert glycerol to 1,3-PD with relatively high yield and productivity (Zeng et al., 1994). Recently, the 1,3-PD pathway from *C. butyricum* was introduced into *C. acetobutylicum*, resulting in a high concentration of 1,3-PD (1104 mM) and a moderate yield (0.65 mol mol^{-1}) (Gonzalez-Pajuelo et al., 2005). Biosynthesis of 1,3-PD from glycerol is associated with the consumption of reducing power NADH, which is generated in the oxidative branch pathway of glycerol. The carbon flux from the oxidation of glycerol is channeled into glycolysis, which leads to the formation of pyruvate-derived by-products. The yield of 1,3-PD depends on the availability of NADH. The maximum supply of NADH would be obtained if acetic acid was the exclusive by-product of the oxidative branch pathway of glycerol (Zeng and Biebl, 2002; Zhang et al., 2005). If biomass formation

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is taken into account and no hydrogen is released, the theoretical maximum yield of 1,3-PD is 0.72 mol mol^{-1} glycerol (Menzel et al., 1997a, b; Zeng and Biebl, 2002). In real fermentation processes, various pyruvate-derived by-products apart from acetic acid are formed (i.e., ethanol, lactic acid). The formation of these by-products competes with the biosynthesis of 1,3-PD for NADH, resulting in a decrease of 1,3-PD yield. Our previous work and other researchers' work all indicated that ethanol is a key competitor to the production of 1,3-PD by *K. pneumoniae* (Menzel et al., 1997a, b; Zhang et al., 2005), and ethanol is a key by-product in the broth of other species of *Klebsiella*, such as *Klebsiella aerogenes* (Streekstra et al., 1997).

Biosynthesis of ethanol from acetyl-CoA is considered as a 2-step process in *K. pneumoniae* under anaerobic conditions (Zeng and Biebl, 2002). Acetyl-CoA is converted to acetaldehyde by NAD^+-dependent aldehyde dehydrogenase (ALDH, encoded by *ald*A gene) and the acetaldehyde is subsequently converted to ethanol by NAD^+-dependent alcohol dehydrogenase (ADH, encoded by the *adh* gene). A straightforward method to improve 1,3-PD yield is to block the formation of ethanol. For this purpose, there are 2 conceivable approaches including inactivation of ALDH or ADH. Surprisingly, these 2 NAD^+-dependent enzymes have received little study in microbes. Considering that ALDH plays a predominant role in ethanol formation, and the *aldA* gene was shown to be highly expressed in *K. pneumoniae* (Ma et al., 2005), inactivation of ALDH is hypothesized to block the formation of ethanol effectively.

In this study, we describe a simple metabolic engineering strategy to improve the production of 1,3-PD by *K. pneumoniae*. A mutant deficient in ALDH activity was generated by insertion of a tetracycline resistance marker into the *aldA* gene. We show that the mutant is unable to produce ethanol, while the production of 1,3-PD is significantly improved. To the best of our knowledge, this is the first report showing that engineering the ethanol pathway has a prominent role in the bacterial production of 1,3-PD.

2. Materials and methods

2.1. Strains, plasmids and culture media

The strains and plasmids used in this study are listed in Table 1. *Eschericha coli* DH5α and its derivatives were routinely grown in LB medium aerobically (37 °C, 180 rpm). *K. pneumonaie* YMU2 and its derivatives were grown in germ medium anaerobically (35 °C, 120 rpm in flasks or 35 °C, 400 rpm in the bioreactor). The germ medium contained (per liter of deionized water): glycerol, 30 g; KH_2PO_4, 1.3 g; $K_2HPO_4 \cdot 3H_2O$, 4.45 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 3.0 g; yeast extract, 1.0 g; $CaCl_2$, 0.003 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and trace elements (Du et al., 2006). When necessary, ampicillin (50 μg ml^{-1}) and/or tetracycline (10 μg ml^{-1}) were added to either the LB medium or the germ medium as selection markers. For cultivating *K. pneumonaie* strains, the pH of the germ medium was maintained at 7.0 with $CaCO_3$ in flasks or by automatic addition of NaOH in the bioreactor. Fed-batch cultivations were carried out in a 5-L bioreactor (Biostat B, B. Braun Biotech International, Germany) with a working volume of 2 L. The glycerol concentration in the medium was maintained at around 20 g L^{-1} by manually regulating the feeding rate of the glycerol solution (800 g L^{-1}).

Table 1
Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
K. pneumoniae YMU2	A low ORP adaptive mutant of *K. pneumoniae* M5aL	Du et al., unpublished
K. pneumoniae DA-1HB	YMU2 *aldA*::*Tcr*	This work
E. coli DH5α		Lab. collection
Plasmids		
pUC18	Amp^r	Lab. collection
pAL1	Amp^r, pUC18 derivative carrying a 1445 bp DNA fragment containing the *aldA* gene	This work
pBR322	Tet^r	Lab collection
pAT1	Amp^r, Tet^r, pAL1 derivative, where a 1.65 kb DNA fragment containing the *Tcr* gene was inserted into the *aldA* gene	This work

[a] Amp^r: ampicillin resistance; Tet^r: tetracycline resistance.

2.2. Construction of plasmids and strains

Total genomic DNA of *K. pneumoniae* YMU2 cells was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA). The *aldA* gene was amplified by PCR using the total genomic DNA of *K. pneumoniae* YMU2 as template DNA and primers A1 and A2, which were designed using the sequence information of the *aldA* gene from *K. pneumoniae* (GenBank accession number AB106869) (Ma et al., 2005):

A1: 5′-GTAGAATTCATGACAGCACCCGTTCAA-CAC-3′,

A2: 5′-GTCAAGCTTCAGGCCTGCAGATAGAC-CAC-3′.

The PCR mixture consisted of 1 ng genomic DNA, 4 nmol each dNTP, 20 pmol each primer, 1 unit Ex. *Taq* DNA polymerase and 2 μl Ex. *Taq* buffer (TaKaRa, Japan) in a total volume of 20 μl. PCR reactions were carried out in PCR Express (Techne, England) unless otherwise stated. Amplification consisted of initial denaturation (94 °C, 5 min); 6 cycles of denaturation (94 °C, 30 s), annealing (62 °C, 90 s), and elongation (72 °C, 90 s); another 25 cycles of denaturation (94 °C, 30 s), annealing

(69 °C, 70 s), and elongation (72 °C, 90 s); and final elongation (72 °C, 10 min). The 1.45-kb PCR product was cloned into pUC18 using the *Eco*RI and *Hin*dIII sites that were introduced by the primers used (underlined), yielding plasmid pAL1 (Fig. 1A). Plasmid pAL1 was extracted from *E. coli* DH5α and subjected to *Pvu*II restriction analysis to confirm the orientation of the inserted *aldA* gene. Subsequent sequencing analysis (Sangon Biotechnology Company, Shanghai, China) using pUC18 universal sequencing primers revealed that the PCR product contained the *aldA* gene.

The tetracycline-resistant gene (*Tcr*) was amplified by PCR using plasmid pBR322 as template DNA and primers P1 and P2:

P1: 5′-ATCCTCGGGAGATCCAGTTCGATGTAAC-3′,

P2: 5′-TGTCACGTATCATTCAGGTCGAGGTG-3′.

The PCR mixture consisted of 0.5 ng plasmid pBR322, 4 nmol each dNTP, 20 pmol each primer, 1 unit Ex. *Taq* DNA polymerase and 2 μl Ex. *Taq* buffer in a total volume of 20 μl. The PCR reaction was carried out at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 67 °C for 45 s, 72 °C for 90 s, and then a final elongation at 72 °C for 10 min. The 1.65-kb PCR product was inserted into the *aldA* gene in pAL1 by using the *Ava*I and *Bas*AI sites that were introduced by the primers used (underlined), yielding plasmid pAT1 (Fig. 1B). The structure of pAT1 extracted from *E. coli* DH5α was confirmed by restriction analysis (*Hin*dIII, *Eco*RI + *Hin*dIII). The inserted *Tcr* gene was sequenced by using sequencing primers flanking the *Tcr* gene. Sequencing results confirmed that no mutations were introduced.

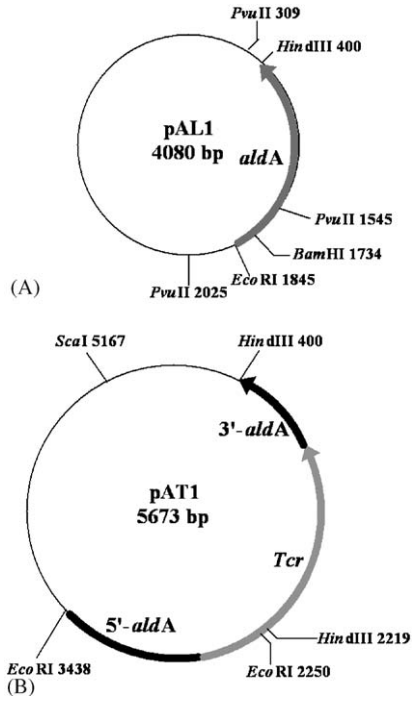

Fig. 1. Structure of the plasmids constructed in this study. Panel (A) pAL1; panel (B) pAT1.

The DNA fragment containing the disrupted *aldA* gene was amplified by PCR using pAT1 as template DNA and primers A1 and A2. The PCR mixture consisted of 0.5 ng pAT1, 4 nmol each dNTP, 20 pmol each primer, 1 unit Ex. *Taq* DNA polymerase and 2 μl Ex. *Taq* buffer in a total volume of 20 μl. The PCR reaction was carried out at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 68 °C for 60 s, 72 °C for 120 s, and then a final elongation at 72 °C for 10 min. The 3.1-kb PCR product was purified and transformed into the competent cells of *K. pneumoniae* YMU2 by standard chemical transformation protocol using 0.1 mol L^{-1} $CaCl_2$ (Sambrook et al., 1989). About 1 mg cells in 200 μl volume was treated with 500 ng DNA and about 80 tetracycline-resistant colonies were obtained. Sixteen of the tetracycline-resistant colonies were subjected to verification of correct recombination and 5 colonies were proved to be correct.

2.3. Preparation of cell-free extracts and ALDH activity assay

K. pneumoniae cells that reached the late exponential phase were harvested by centrifugation from 4 ml culture (20,000*g* for 2 min at 4 °C) using a centrifuger (KUBOTA 6930, Japan). The cell pellets were washed with a cold solution of 50 mM glycine/NaOH buffer (pH 9.5) and resuspended in 4 ml glycine/NaOH buffer (pH 9.5). The cells were disrupted ultrasonically at 4 °C for 10 min with 3-s pulses at 200 W by a cell sonicator (SCIENTZ JY92-II, China). Cell debris was removed by centrifugation (20,000*g* for 20 min at 4 °C), resulting in a cell-free extract (CFE), which was subsequently used for ALDH activity assay. Protein concentrations of CFE were determined by the Bradford method (Broadford, 1976) using bovine serum albumin as a standard.

According to Sanchez (1998), an assay of ALDH (CoA-acetylating) activity in the direction of acetaldehyde formation can be measured only with the purified enzyme, since crude extracts contain very active NADH oxidase activity, which interferes with the assay. Therefore, the reverse reaction, in the direction of acetaldehyde oxidation, was used to determine the ALDH activity in this study. The reaction was carried out at 30 °C for 10 min in the presence of 2 mmol L^{-1} acetaldehyde, 0.2 mmol L^{-1} CoA-SH, and 0.8 mmol L^{-1} NAD^+ in 50 mmol L^{-1} glycine/NaOH buffer, pH 9.5. The activity was calculated from the linear slope of increasing absorption of NADH at 340 nm using $\varepsilon = 6220\ L\ mol^{-1}\ cm^{-1}$. One unit of enzyme activity is defined as the amount of protein that produces 1 pmol NADH per minute.

2.4. Assay of biomass and metabolic products

Biomass was determined by measuring turbidity at 650 nm using a UV–visible spectroscopy system (Agilent 8453, Germany) and by cell dry weight (CDW) method. The CDW could also be calculated from OD_{650} using an

equation that CDW ($g\,L^{-1}$) = $0.255 \times OD_{650} - 0.0176$. The concentrations of glycerol, 1,3-PD, lactic acid, ethanol, acetic acid, 2,3-butanediol and succinic acid were determined by HPLC (Du et al., 2006). The extracellular oxidoreduction potential (ORP) level was determined by ORP electrode and intracellular concentrations of NAD^+ and NADH were also determined by the procedure described in our previous work (Du et al., 2006).

3. Results

3.1. Inactivation of ALDH in K. pneumoniae YMU2

Homologous recombination technique was used to inactivate the ALDH in *K. pneumoniae* YMU2. The *aldA* gene from *K. pneumoniae* YMU2 was cloned into pUC18, then disrupted by inserting a tetracycline resistance gene *Tcr* into the *Ava*I and *Bsa*AI sites of the *aldA* gene. The restriction sites *Ava*I and *Bsa*AI were located in the 828th bp and the 877th bp of the *aldA* gene, respectively. After inserting the *Tcr* gene, the length of the *aldA* gene fragments flanking the *Tcr* gene were 828 bp upstream of the 5′-*Tcr* and 568 bp downstream of the *Tcr*-3′. This ensured homologous recombination in *K. pneumoniae* could occur in a relatively high efficiency.

The PCR-amplified 3.1-kb DNA fragment, corresponding to *aldA*-5′-*Tcr*-3′-*aldA*, was transformed into *K. pneumoniae* YMU2. Tetracycline-resistant colonies were screened by colony PCR using primers A1 and A2. A 1.45-kb amplicon and a 3.1-kb amplicon represent wild-type and putative mutant, respectively. After screening for tetracycline-resistant colonies, a putative *aldA* knockout mutant, *K. pneumoniae* DA-1HB, was obtained (Fig. 2). The PCR product shown in lane DA of Fig. 2 was purified and subjected to restriction analysis and sequencing analysis, confirming that the *Tcr* gene has inserted into the *aldA* gene of *K. pneumoniae* (data not shown).

The ALDH activities of the wild-type strain YMU2 and the mutant DA-1HB were determined. The specific activity of ALDH in the CFE of *K. pneumoniae* DA-1HB was around the lowest detection threshold (Fig. 3). This confirmed that the *aldA* gene in *K. pneumoniae* DA-1HB was disrupted, resulting in an inactivation of ALDH.

3.2. Effect of ALDH inactivation on cell growth of K. pneumoniae

Growth of the parent strain YMU2 and the mutant strain DA-1HB, as well as the associated ORP of the culture broth, were monitored in parallel fed-batch cultures, as shown in Fig. 4. Both the maximum growth rate of the mutant strain DA-1HB ($0.13\,g\,L^{-1}\,h^{-1}$) and the growth yield ($2.33\,g\,L^{-1}$) were lower than those of the parent strain YMU2 ($0.18\,g\,L^{-1}\,h^{-1}$ and $3.42\,g\,L^{-1}$, respectively). This clearly indicated that the cell growth of *K. pneumoniae* was impaired due to inactivation of ALDH. At the same time, the change of ORP of the mutant strain

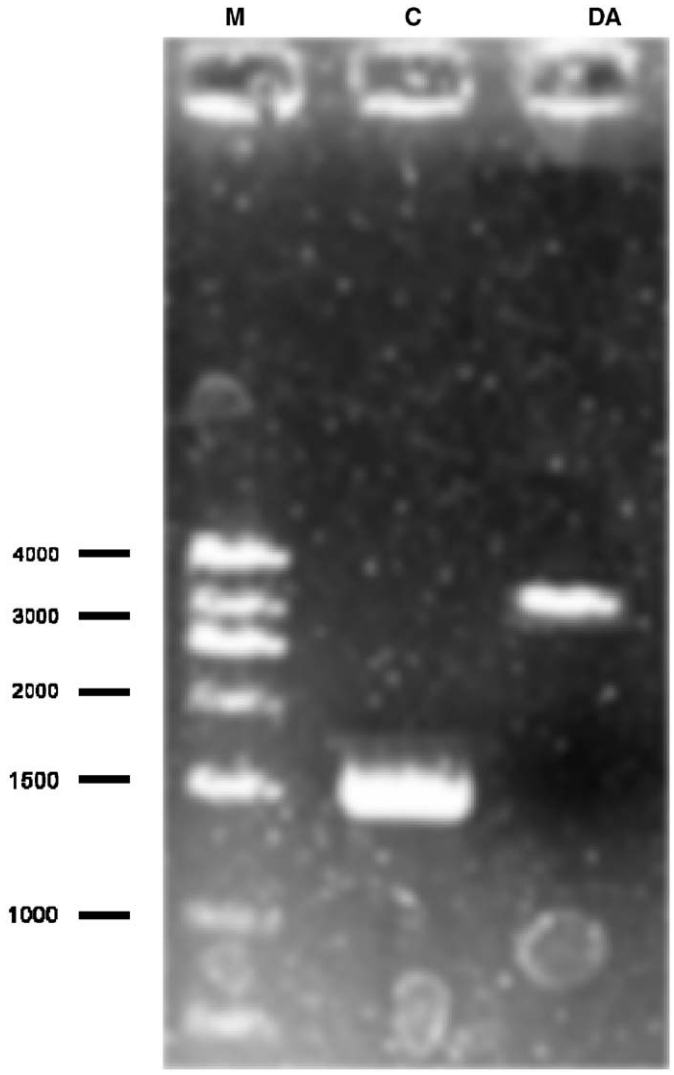

Fig. 2. Agarose gel electrophoresis of PCR products generated using *K. pneumoniae* colonies and primers A1 and A2. Lanes: M, DNA marker DGL 4000 (bp); C, *K. pneumoniae* YMU2; DA, *K. pneumoniae* DA-1HB.

decreased from 80 to −225 mV in the first 20 h of fermentation, gentler than that of the parent strain. After 20 h of fermentation, the ORP of the mutant strain maintained at -225 ± 10 mV, lower than that of the parent strain which increased from −240 to -210 ± 5 mV. The mutant strain displayed a relatively lower level of ORP, suggesting that the ratio of reductive components to oxidative components in the fermentation broth increased.

3.3. ALDH inactivation triggers metabolic flux redistribution in K. pneumoniae

Metabolite spectra of the parent strain YMU2 and the mutant strain DA-1HB under standard 1,3-PD fermentation conditions were determined (Fig. 5). Besides the target product 1,3-PD, the main by-products converted from glycerol by *K. pneumoniae* DA-1HB included acetic acid, lactic acid, minor amounts of 2,3-butanediol, ethanol and succinic acid. This spectrum was distinguished from that of

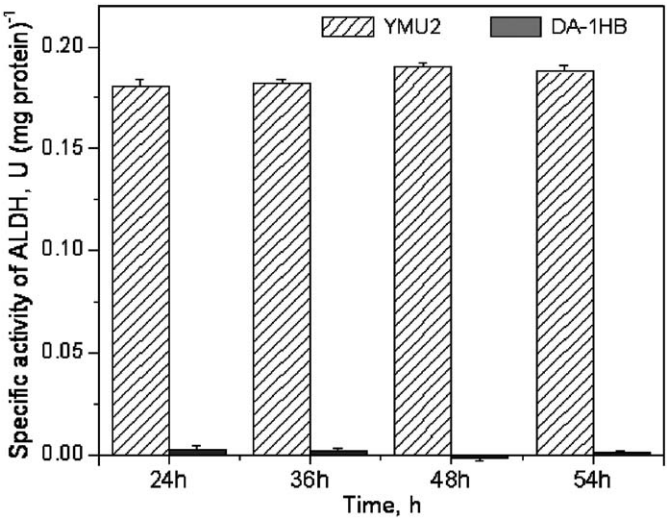

Fig. 3. Time course of ALDH activity determination in the mutant strain *K. pneumoniae* DA-1HB and the parent strain *K. pneumoniae* YMU2. Fed-batch cultivations were carried out in a 5-L bioreactor using germ medium as described in Section 2. The culture was aerated with nitrogen ($0.8\,L\,L^{-1}\,min^{-1}$) to maintain anaerobic conditions. The glycerol concentrations in these fermentations were maintained at around $20\,g\,L^{-1}$ by using the same glycerol feeding profile determined based on preliminary experiments.

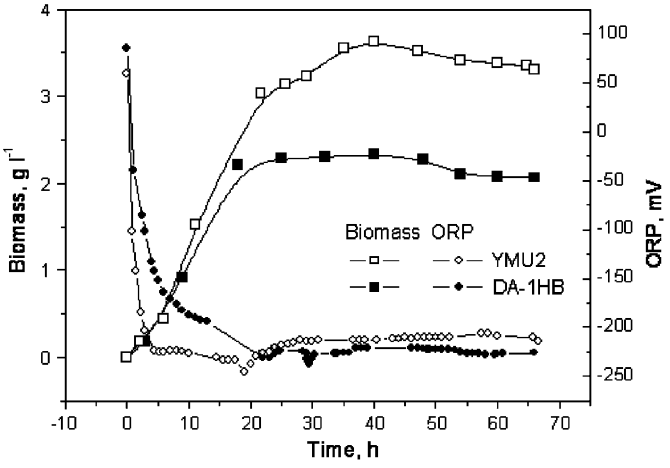

Fig. 4. Time course of cell growth and ORP of the culture broth of the mutant strain *K. pneumoniae* DA-1HB and the parent strain *K. pneumoniae* YMU2. Fed-batch cultivations were carried out in a 5-L bioreactor using germ medium as described in Section 2. The culture was aerated with nitrogen ($0.8\,L\,L^{-1}\,min^{-1}$) to maintain anaerobic conditions. The glycerol concentrations in these fermentations were maintained at around $20\,g\,L^{-1}$ by using the same glycerol feeding profile determined based on preliminary experiments.

the parent strain *K. pneumoniae* YMU2, which comprised of ethanol, acetic acid, minor amounts of lactic acid, 2,3-butanediol and succinic acid. Inactivation of ALDH resulted in a nearly abolished ethanol formation but a significantly improved 1,3-PD production. The final titer of 1,3-PD and ethanol were 927.6 and 36.8 mmol L^{-1}, respectively, which were 132.8% and 18.3% of those of the parent strain.

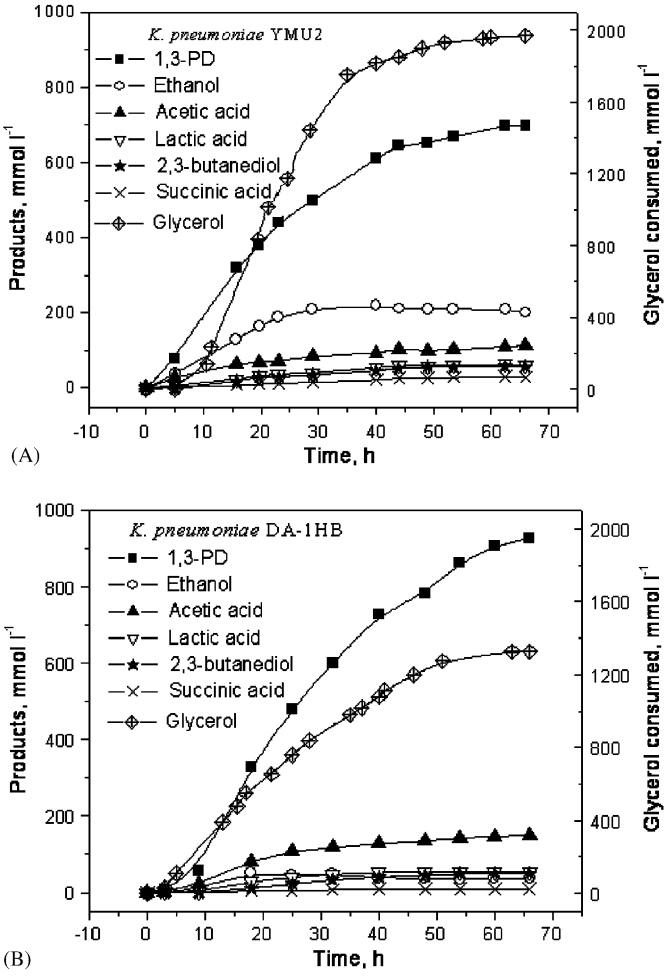

Fig. 5. Metabolites profiles of the mutant strain *K. pneumoniae* DA-1HB (panel A) and the parent strain *K. pneumoniae* YMU2 (panel B). Fermentation conditions were the same as described in the legend of Fig. 4.

Differences between fermentation parameters of the parent strain and the mutant strain were calculated and shown in Table 2. Clearly, ALDH inactivation triggered metabolic flux redistribution in *K. pneumoniae*. Apart from the increased titer of 1,3-PD, the final titer of acetic acid in the mutant strain DA-1HB was 131.2% of that of the parent strain. While the lactic acid and 2,3-butanediol formation were comparable, the growth yield, ethanol concentration, succinic acid concentration and glycerol consumption of the mutant strain DA-1HB were only 68.1%, 18.3%, 34.7% and 67.4% of those of the parent strain. Due to its lower growth yield, the specific 1,3-PD-producing capability (1,3-PD produced per gram of cells) of the mutant strain DA-1HB was 2-fold that of the parent strain. The productivity of 1,3-PD and the yield of 1,3-PD of the mutant strain reached 14.05 mmol $L^{-1}\,h^{-1}$ and 0.699 mol mol^{-1}, almost double that of the parent strain and close to the maximum theoretical yield. Production of 1,3-PD by the mutant strain DA-1HB with ALDH inactivated was superior under these fermentation conditions.

Table 2
Comparing of the fermentation of the mutant strain *K. pneumoniae* DA-1HB with those of the parent strain *K. pneumoniae* YMU2

Strains	*K. pneumoniae* DA-1HB	*K. pneumoniae* YMU2	Ratio of DA-1HB to YMU2 (%)
Fermentation time (h)	66	66	—
Average specific activity of ALDH (μmol min^{-1} (g protein)$^{-1}$)	0	0.181	—
Biomass (g L^{-1})	2.33	3.42	68.1
Glycerol consumed (mmol L^{-1})	1327.8	1970.5	67.4
1,3-PD (mmol L^{-1})	927.6	698.6	132.8
Ethanol (mmol L^{-1})	36.8	201.2	18.3
Acetic acid (mmol L^{-1})	149.8	114.2	131.2
Lactic acid (mmol L^{-1})	54.9	60.1	91.3
2,3-Butanediol (mmol L^{-1})	49.4	56.6	87.3
Succinic acid (mmol L^{-1})	9.6	27.7	34.7
Specific 1,3-PD producing capability (mmol (g cells)$^{-1}$)	398.1	204.3	194.9
Productivity of 1,3-PD (mmol L^{-1} h^{-1})	14.05	10.58	132.8
Yield of 1,3-PD (mol mol^{-1})	0.699	0.355	196.9

According to the time course of cell growth (Fig. 4), the whole fermentation process can be divided into 2 phases—growth phase and non-growth phase. As shown in Fig. 6, a prominent flux shift toward the production of 1,3-PD was observed in the fermentation using the mutant strain DA-1HB, especially in the growth phase (0–18 h for DA-1HB vs. 0–35 h for YMU2). Glycerol flux to the reductive branch increased, while the flux of glycerol to the oxidative branch reduced significantly. In addition, the individual fluxes to various pyruvate-derived by-products redistributed remarkably in mutant strain DA-1HB. In the growth phase, glycerol fluxes to ethanol and 2,3-butanediol decreased obviously and those to acetic acid and lactic acid increased accordingly, compared with those of the parent strain. And in the non-growth phase, glycerol fluxes to all by-products decreased significantly. The redistribution of major metabolic flux in *K. pneumoniae* DA-1HB led to a significant increased yield of 1,3-PD.

Interestingly, a significant increase in the ratio of intracellular NADH to NAD^+ was observed (Fig. 7). This might have resulted from the retarded oxidation of NADH due to the inhibitory effect of ALDH inactivation on ethanol formation and subsequently resulted in a shift toward the production of 1,3-PD for the fulfillment of the redox balance in vivo.

4. Discussion

Production of 1,3-PD from glycerol is a model oxidoreduction-coupled biosynthesis process. The NADH required for the biosynthesis of 1,3-PD is mainly regenerated from NAD^+ along with the oxidation of glycerol. As one of the major by-products, the formation of ethanol competes for NADH and is therefore unfavorable for production of 1,3-PD by *K. pneumoniae*. We hypothesized that inactivation of ALDH, the upriver enzyme in the ethanol formation pathway, would block the formation of ethanol, and thus improve the yield of 1,3-PD.

For the first time, the ALDH of *K. pneumoniae* was inactivated by disrupting the coding gene *aldA*. We presented evidence that the inactivation of ALDH not only led to a dramatic decrease of ethanol production, but also triggered a pronounced redistribution of metabolic fluxes. Along with the decrease of ethanol formation, production of 1,3-PD and acetic acid were improved significantly. In addition, production of succinic acid was weakened and final titers of lactic acid and 2,3-butanediol were not altered remarkably. When the mutant *K. pneumoniae* with ALDH inactivated was fermented in the germ medium with glycerol as the sole carbon source for 66 h, the final titer and productivity of 1,3-PD reached 927.6 mmol L^{-1} and 14.05 mmol L^{-1} h^{-1} respectively. Most importantly, due to more efficient consumption of glycerol in the mutant strain DA-1HB, the yield of 1,3-PD from glycerol was increased from 0.355 to 0.699 mol mol^{-1}, which is 97.1% of the theoretical maximum yield. The final titer of ethanol was 36.8 mmol L^{-1}, only 18.3% of that of the parent strain. Although the major pathway for ethanol production was disrupted, the production of a minor amount of ethanol could be due to the role of other dehydrogenases whose functions were not annotated but have catalytic activities to convert thioester to aldehyde.

Inactivation of ALDH affected the ratio of NADH to NAD^+ in vivo and the ORP in vitro. The dramatically decreased formation of ethanol due to ALDH inactivation in *K. pneumoniae* DA-1HB led to a significantly increased ratio of NADH to NAD^+. Consequently, glycerol flux was shifted toward the production of 1,3-PD under anaerobic conditions for the fulfillment of the redox balance in vivo. In addition, as shown in Fig. 6, the production of acetic acid by the mutant was strengthened because its branch process, ethanol formation (ethanol ← acetyl-CoA → acetic acid), was weakened. Carbon fluxes to lactic acid and 2,3-butanediol were slightly increased in the mutant, which is presumably due to the fact that NADH availability increased. However, the increased NADH availability in the mutant did not increase the carbon flux to succinic acid, which is also a by-product competing for NADH. This may be due to the fact that the formation of succinic acid is ADP dependent. The significantly increased production of acetic acid in the mutant may decrease the availability of ADP, and therefore reduce the formation of succinic acid (Biebl et al., 1999). Due to the alteration of metabolism in *K. pneumoniae*, the fraction of oxidative components in the culture broth was decreased, which led to a decrease in ORP in vitro.

The increase of NADH/NAD^+ ratio in vivo and the decrease of ORP in vitro resulted from ALDH inactivation

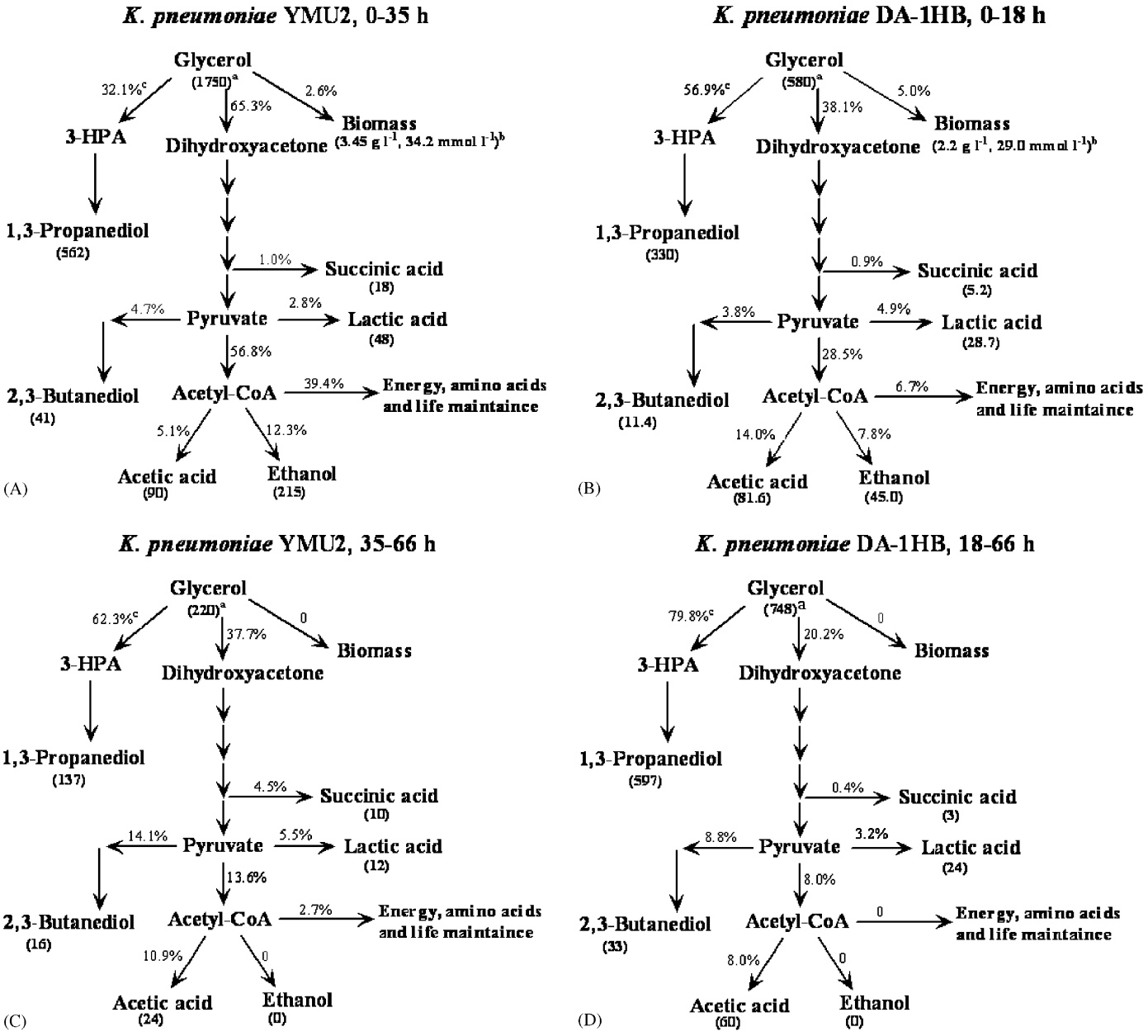

Fig. 6. Metabolic fluxes of glycerol in the mutant strain *K. pneumoniae* DA-1HB and the parent strain *K. pneumoniae* YMU2. Superscripts: a, final molar titer of glycerol consumed (mmol L^{-1}), as are those of products produced; b, yield of biomass, the molar concentration is calculated based on previous work (Zeng et al., 1993); c, molar fractions of glycerol fluxes in overall glycerol consumed.

comprised of the micro- and macro-environments for cell growth and metabolism. Therefore, a remarkable redirection of intracellular metabolic flux, as shown in Fig. 6, is conceivable. Based on the metabolic network in Zeng et al. (1993), in the non-growth phase during the anaerobic culture of both the ALDH-deficient strain DA-1HB and the parent strain YMU2, glycerol consumed was almost transformed into 1,3-PD and by-products. But in the growth phase of DA-1HB fermentation, except for glycerol consumed for 1,3-PD production (56.9%, molar fraction), for by-products (31.4%) and for biomass (5.0%), the residual 6.7% of glycerol consumed was metabolized in unknown pathways, different from the parent strain with 39% of glycerol consumed was metabolized in these pathways. The metabolic pathways for assimilating the residual glycerol (the glycerol which was not used for the formation of 1,3-PD, by-products, or biomass) remain unknown. From Fig. 5, it was shown that the glycerol consumption rate was high in the growth phase. And the imbalanced carbon distribution only occurred in the growth phase (Fig. 6). This could be due to 3 reasons: (1) glycerol was transformed into other building blocks, such as amino acids, along with cell growth. Some of them were used for biomass synthesis and others were accumulated along with the fast cell growth. As we did not determine the concentration of free amino acids in the broth, the

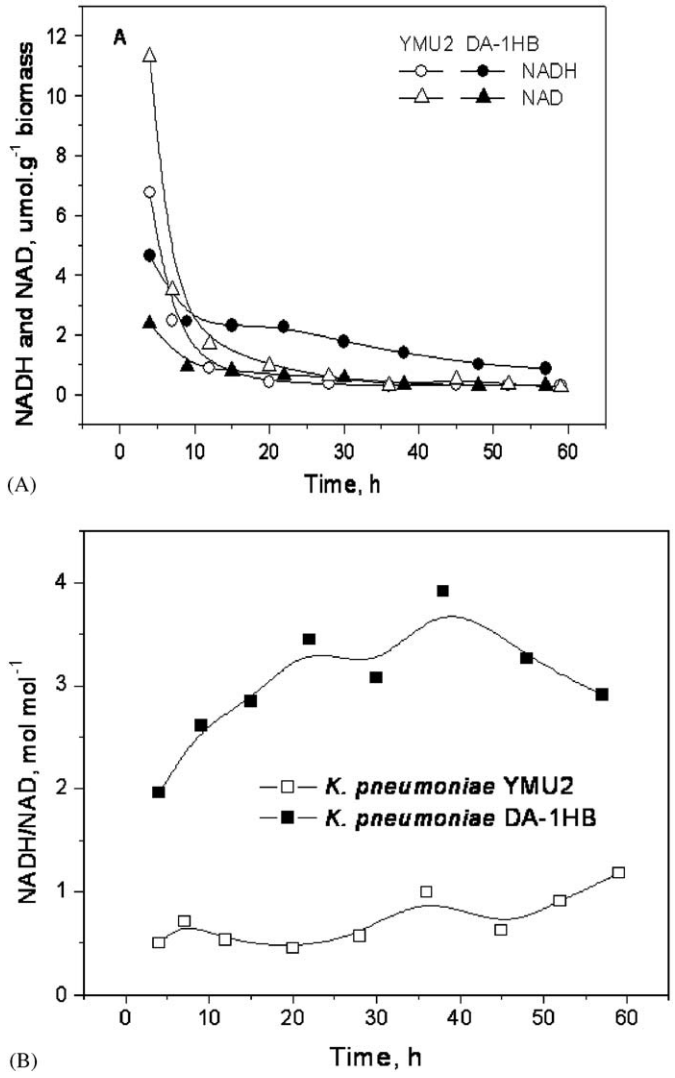

Fig. 7. Effect of ALDH inactivation on the ratio of intracellular NADH to NAD^+ in *K. pneumoniae* strains.

contribution of glycerol consumption to the formation of these building blocks could not be evaluated. (2) *K. pneumoniae* can simultaneously use the 2 enzymes—pyruvate dehydrogenase and pyruvate: formate lyase for anaerobic cleavage of pyruvate in the glycerol fermentation (Menzel et al., 1997a, b). Formate and CO_2 would be formed if the latter enzyme was preponderant. This may also account for the low carbon balance in the wild-type strain. (3) Since part of the TCA cycle reaction is active in the anaerobic glycerol fermentation (Zeng and Biebl, 2002), it would be possible that part of the glycerol was consumed in this way. On the other hand, a balanced carbon distribution of the mutant strain DA-1HB was observed in the growth phase. Disruption of ALDH resulted in an increased $NADH/NAD^+$ ratio, which was inhibitory for cell growth and glycolysis. Due to its higher growth rate and more biomass, the parent strain YMU2 would consume more glycerol in the 3 possible pathways than its mutant strain DA-1HB. This resulted in retarded glycerol consumption and a relatively balanced carbon distribution. Further metabolomics study would reveal the truth why the carbon balance of the wild-type strain is much lower than the mutant strain in the growth phase.

Inactivation of ALDH decreased biomass production, as shown in Fig. 3. This could be due to, in one aspect, the inhibitory effect of the increased $NADH/NAD^+$ ratio in vivo and the decreased ORP in vitro on cell growth. Cell growth also produces NADH; obviously, the accumulation of NADH is not favorable for synthesizing cell materials. In another aspect, the increased production of acetic acid may also contribute to the inhibitory effect on cell growth. Acetic acid was the strongest toxic compound as compared with the other products (1,3-PD, ethanol, lactic acid). The inhibition potential of acetic acid on the growth of *K. pneumoniae* has been studied (Zeng et al., 1994). They found that the maximum concentration of undissociated acetic acid that the strain tolerated was $0.35\,g\,L^{-1}$ ($5.83\,mmol\,L^{-1}$). Our previous study also showed that significant inhibition on cell growth was observed upon addition of $100\,mmol\,L^{-1}$ external acetic acid. Although the production of acetic acid can generate additional ATP, the inhibition effect on cell growth is a disadvantage for 1,3-PD production, which is coupled with cell growth. In order to further improve the titer and productivity of 1,3-PD by the mutant strain, measures must be taken to eliminate the inhibition of acetic acid, or to decrease the production of acetic acid by other engineering approaches.

In conclusion, inactivation of ALDH in *K. pneumoniae* is a successful engineering approach to improve the production of 1,3-PD. For the first time, the final titer of 1,3-PD produced by *K. pneumoniae* reached $70.5\,g\,L^{-1}$ (927.6 mM) and the simultaneous yield of 1,3-PD from glycerol reached $0.699\,mol\,mol^{-1}$. Our study also suggested a potential useful approach to improve the efficiency of analogous bioprocesses coupled with oxidoreductive reactions, such as biosynthesis of glycerol (Petrovska et al., 1999; Wang et al., 2001), pyruvic acid (Garrigues et al., 2001) and sorbitol (Ferraz et al., 2001; Viikari, 1984), which were limited by the availability of coenzyme including NADH and NAD^+.

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