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Enhancement of 1,3-propanediol production by expression of pyruvate decarboxylase and aldehyde dehydrogenase from *Zymomonas mobilis* in the acetolactate-synthase-deficient mutant of *Klebsiella pneumoniae*

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Abstract The acetolactate synthase (*als*)-deficient mutant of *Klebsiella pneumoniae* fails to produce 1,3-propanediol (1,3-PD) or 2,3-butanediol (2,3-BD), and is defective in glycerol metabolism. In an effort to recover production of the industrially valuable 1,3-PD, we introduced the *Zymomonas mobilis* pyruvate decarboxylase (*pdc*) and aldehyde dehydrogenase (*aldB*) genes into the *als*-deficient mutant to activate the conversion of pyruvate to ethanol. Heterologous expression of *pdc* and *aldB* efficiently recovered glycerol metabolism in the 2,3-BD synthesis-defective mutant, enhancing the production of 1,3-PD by preventing the accumulation of pyruvate. Production of 1,3-PD in the *pdc*- and *aldB*-expressing *als*-deficient mutant was further enhanced by increasing the aeration rate. This system uses metabolic engineering to produce 1,3-PD while minimizing the generation of 2,3-BD, offering a breakthrough for the industrial production of 1,3-PD from crude glycerol.

Keywords *Klebsiella pneumoniae* · Glycerol · 1,3-propanediol · Acetolactate synthase · Metabolic engineering

The authors S.-M. Lee and W.-K. Hong are co-first authors and contributed equally.

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Introduction

Copious raw glycerol is formed as the main by-product of biodiesel production, corresponding to as much as 10 % (w/w) of the generated biodiesel [1]. This surplus of raw glycerol poses a significant environmental problem, since it cannot be discharged directly into the environment without any treatment [2]. Numerous researchers have sought to use glycerol as a low-cost feedstock for refinement into industrially valuable materials [3–5], including 1,3-propanediol (1,3-PD). 1,3-PD is a valuable chemical that is mainly polymerized with terephthalates for the synthesis of polymethylene terephthalates, which are used in the manufacturing of textile fiber, film and plastic [6].

Klebsiella pneumoniae is a typical microbial strain that is capable of producing 1,3-PD from glycerol. The metabolic pathway responsible for microbial conversion has been well studied (Fig. 1) [7]. In *K. pneumoniae*, glycerol is used as a carbon and energy source for the generation of biomass, resulting in the production of various metabolites, including acetate, ethanol, lactate and 2,3-butanediol (2,3-BD) (Fig. 1). Additionally, a reductive pathway was accompanied to balance intracellular redox level during glycerol assimilation. In the reductive pathway, 1,3-PD is generated from glycerol by the action of reduced nicotinamide adenine dinucleotide (NADH)-dependent oxidoreductase).

Based on the genetic elucidation of fermentative glycerol metabolism [8], researchers have sought to enhance the production yield of 1,3-PD [9–13]. One efficient strategy for increasing 1,3-PD production is engineering the

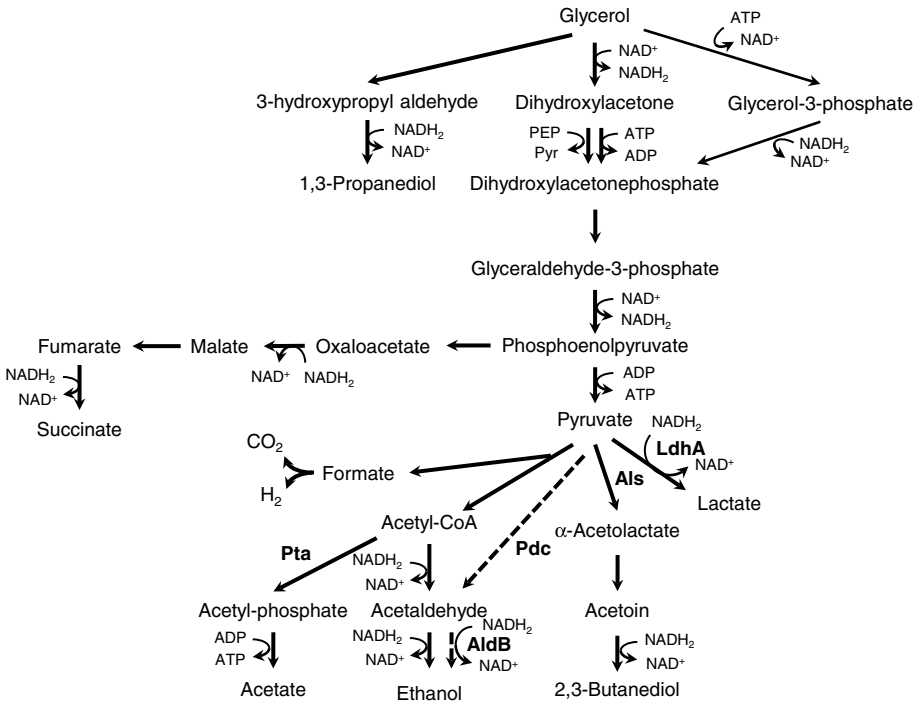

Fig. 1 The glycerol metabolic pathway in *Klebsiella pneumoniae*. Pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (AldB) from *Zymomonas mobilis* are indicated in *dotted line*. *LdhA* lactate dehydrogenase, *Als* acetolactate synthase, *Pta* phosphate acetyltransferase

oxidative pathway to minimize the production of by-products. A successful result was obtained following inactivation of the lactate dehydrogenase (*ldhA*) gene, which is involved in lactate synthesis and is a major metabolite of the glycerol oxidation pathway [14]. We previously inactivated 2,3-BD production and assessed subsequent glycerol metabolism in *K. pneumoniae* [14]. This genetic engineering reduced the production level of 1,3-PD, and was thus unsatisfactory for industrial applications. Given that the boiling point of 2,3-BD is similar to that of 1,3-PD [15], potentially hampering the high-purity recovery of 1,3-PD, strategies should be developed to maximize the production of 1,3-PD while minimizing that of 2,3-BD. Here, we successfully used genetic engineering to enhance the production of 1,3-PD in a 2,3-BD-deficient mutant.

Materials and methods

Bacterial strains, plasmids, and growth media

The *K. pneumoniae* Δ*ldhA* and Δ(*ldhA als*) mutant, derived from ATCC 200721, was described previously [14]. *Escherichia coli* DH5α was used for DNA manipulation. The λ Red and FLP recombinases were expressed by helper plasmids pKD46 and pCP20, respectively. The replication of these plasmids is temperature-sensitive, allowing them to be easily eliminated. The pIJ773 vector was used as the source of the apramycin-resistance gene. The pBR322 vector was used to create pBR-*aldB-pdc*, which encoded the *Zymomonas mobilis* pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (AldB) genes. Microbial cells were grown in LB [yeast extract (Difco), 0.5 % (w/v); Bacto-tryptone (Difco), 1.0 % (w/v); and NaCl, 1.0 % (w/v)] or germ medium [16] supplemented with appropriate antibiotics [ampicillin (50 μg mL^{-1}), apramycin (50 μg mL^{-1}), or tetracycline (10 μg mL^{-1})]. The germ medium contained 20 g m^{-1} crude glycerol (purity 80 % w/w), 2 g L^{-1} $(NH_4)_2SO_4$, 3.4 g L^{-1} K_2HPO_4, 1.3 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4$, 0.02 g L^{-1} $CaCl_2{\cdot}2H_2O$, 1 g L^{-1} yeast extract, 1 mL Fe solution [5 g L^{-1} $FeSO_4{\cdot}7H_2O$ and 4 mL L^{-1} HCl (37 %, w/v)], and 1 mL trace element solution [70 mg L^{-1} $ZnCl_2$, 100 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 60 mg L^{-1} H_3BO_3, 200 mg L^{-1} $CoCl_2{\cdot}4H_2O$, 20 mg L^{-1} $CuCl_2{\cdot}2H_2O$, 25 mg L^{-1} $NiCl_2{\cdot}6H_2O$, 35 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, and 4 mL L^{-1} HCl (37 %, w/v)]. Crude glycerol was obtained from a biodiesel-producing company (GSBio, Yeosu, Korea).

Deletion of the *pta* gene

For construction of the *pta* deletion mutant (Supplementary Fig. 1), the 300-bp DNA sequences located upstream and downstream of *pta* were PCR amplified using oligonucleotides P1 (5′-acccgcaataattcgagctg-3′) and P2 (5′-gagcgctgtaccgctttgta*GTTAAC*gcgataggtttaaagacgctcag-3′; italics indicate an *Hpa*I site) for the upstream region, and P3 (5′-ctgagcgtcttttaaacctatcgc*GTTAAC*tacaaagcggtacagcgctc-3′; italics indicate an *Hpa*I site) and P4 (5′-agagagcgagcgcgaataaa-3′) for the downstream region. The PCR products were annealed via overlapping regions of the P2 and P3 primers, amplified as

a single fragment using primers P1/P4, and cloned into the pGEM-T Easy vector. The resulting plasmid was digested with *Hpa*I and ligated with an apramycin-resistance gene [*aac(3)IV*] obtained from pIJ773 by digestion with *Eco*RI and *Hin*dIII and treatment with the Klenow fragment. The resultant plasmid, designated pT-pta-Apra, was used as a template for PCR amplification of the deletion cassette, which was introduced into *K. pneumoniae* Δ(*ldhA als*) by electroporation [17] to induce homologous recombination. Correct integration of the DNA fragment was confirmed by Southern hybridization using the upstream regions of *pta* and *aac(3)IV* to probe *Dra*I-digested chromosomal DNA.

Construction of recombinant strain expressing *pdc* and *adh* gene

The 1.8-kb open reading frame (*orf*) of *pdc* was amplified from chromosomal DNA of *Z. mobilis* ZM4 using the following primers: Ppdc-F (5′-*TCTAGA*ATGAGTTATACTGTCGGTACCTATTTAGC-3′; italicized bases indicate an *Xba*I site) and Ppdc-R (5′-*CTCGAG*CTGCAGCTAGAGGAGCTTGTTAACAGGCTTAC-3′; italicized and underlined letters indicate an *Xho*I and a *Pst*I site, respectively). A DNA fragment including a 1.15-kb *aldB* segment was amplified from chromosomal DNA of *Z. mobilis* ZM4 using the following primers: PaldB-F (5′-*AGATCT*ATGGCTTCTTCAACTTTTTATATTCC-3′; italicized letters indicate a *Bgl*II site) and PaldB-R (5′-*CTCGAG*TCTAGATTAGAAAGCGCTCAGGAAGAGTT-3′; italicized and underlined letters indicate *Xho*I and *Xba*I sites, respectively). The *lacZ* promoter sequence (P_{lacZ}-*aldB*) was amplified using specific primers; these were PlacZ-aldB-F (5′-*GAATTC*AGCGGGCAGTGAGCGCAA-3′; italicized letters indicate an *Eco*RI site) and P_{lacZ}-aldB-R (5′-*CTCAGA*AGATCTAGCTGTTTCCTGTGTGAAATTG-3′; italicized and underlined letters indicate *Xho*I and *Bgl*II sites, respectively). Amplified DNA fragments were cloned into the pGEM TEasy (Promega) vector, followed by nucleotide sequencing to confirm the absence of any sequence error. A *Bgl*II–*Xho*I fragment including the *aldB* gene was inserted between equivalent restriction sites downstream of a *lacZ* promoter sequence. An *Xba*I–*Xho*I fragment, including the *pdc* gene, was next inserted between corresponding sites of pGEM-P_{lacZ}-*aldB* to create pGEM-P_{lacZ}-*aldB-pdc*. Finally, an *Eco*RI–*Pst*I fragment including P_{lacZ}-*aldB-pdc* was inserted between the corresponding sites of pBR322 to create pBR-*aldB-pdc*. The final plasmids was transformed into *K. pneumoniae* Δ(*ldhA als*) by electroporation.

Preparation of cell-free extracts and enzyme activity assays

Cells were grown in culture and shaken overnight at 100 rpm in 250-mL flasks containing 50 mL of glycerol media, until they reached the stationary phase. The cells were then harvested by centrifugation (13,000 rpm, 4 °C, 10 min), and each cell pellet was washed twice with cold potassium phosphate buffer (50 mM, pH 7.0) and re- suspended in potassium phosphate buffer (50 mM, pH 7.0). The samples were sonicated in an ice bath for 90 cycles (each cycle = 3 s at 200 W followed by a 5-s pause). Cell debris was removed by centrifugation (13,000 rpm, 4 °C, 20 min), and the enzyme activities of pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (AldB) were measured as described by Postma et al. [18]. One unit of enzyme activity was defined as the amount of enzyme that consumed 1 μmol of substrate per min. Protein concentrations were determined by a protein assay kit (Bio-Rad), with BSA used as a standard. All activity measurements were performed in triplicate.

Fermentation by *K. pneumoniae* strains

For fermentations, seed cells were prepared in 1-L flasks containing 200 mL of germ medium. The flasks were incubated at 37 °C for 12 h, and then the culture [10 % (v/v)] was inoculated into the growth vessel. Fed-batch fermentations were conducted in a 5-L stirred-vessel system (Kobiotech Co. Ltd.) containing 2 L of germ medium; all fermentation experiments were conducted at 37 °C with stirring at 200 rpm. Unless stated otherwise, the pH was maintained at pH 6.5 ± 0.2 and the aeration rate was 2.0 vvm. The crude glycerol was controlled between 30 and 80 g L^{-1} in subsequent fed-batch fermentations. All data are given as the average from three independent experiments.

Metabolite analysis

The levels of residual glycerol 1,3-PD, ethanol, acetate, lactate, succinate, and 2,3-BD were determined using an HPLC apparatus equipped with a refractive index detector and an organic acid analysis column (300 × 78 mm; Aminex HPX-87H; Bio-Rad). The mobile phase was 5 mM H_2SO_4 and the flow rate was 0.6 mL min^{-1}. The column and cell temperatures were 65 and 45 °C, respectively. The biomass concentration was determined by measurement of optical density at 600 nm (OD_{600}).

Results

Effect of *pta* gene deletion in the *als*-deficient *K. pneumoniae* mutant

We previously showed that inactivation of the acetolactate synthase (Als) gene reduces 2,3-BD production and subsequent 1,3-PD production in *K. pneumoniae* [14]. Here, we found that glycerol assimilation and cell growth were also

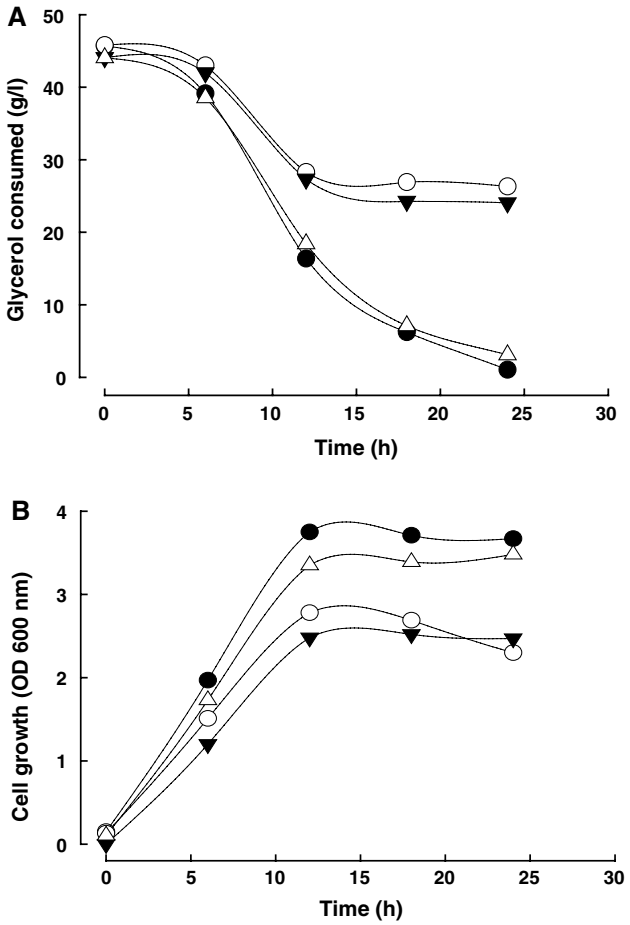

Fig. 2 Glycerol consumption rate (**a**) and cell growth (**b**) of *K. pneumoniae* mutants. Symbols: *closed circles*, *K. pneumoniae* Δ*ldhA*; *open circles*, *K. pneumoniae* Δ(*ldhA als*); *closed triangles*, *K. pneumoniae* Δ(*ldhA als pta*); and *open triangles*, *K. pneumoniae* Δ(*ldhA als*) harboring pBR-*aldB-pdc*

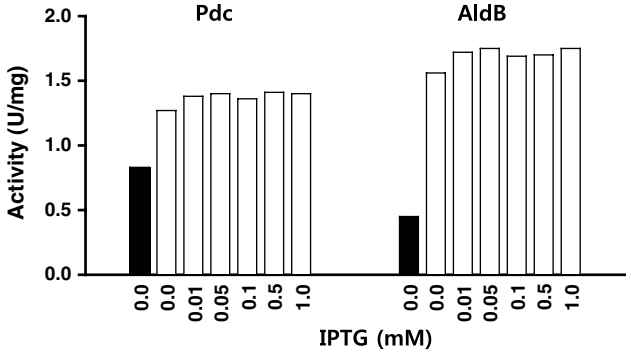

Fig. 3 Effect of IPTG concentration on the enzyme activity. Symbols: *closed bar* crude cell lysate from *K. pneumoniae* Δ(*ldhA als*), and *open bar* crude cell lysate from Δ(*ldhA als*)/pBR-*aldB-pdc*. *Pdc* pyruvate decarboxylase, *AldB* aldehyde dehydrogenase

decreased in the mutant (Fig. 2), and confirmed the low-level production of 1,3-PD (Table 1). We also observed a remarkable increase in the acetate level of the *als*-deficient mutant (Table 1), prompting us to speculate that there may be an association between acetate accumulation and the observed defect in glycerol metabolism. To test this hypothesis, we constructed and characterized a phosphate acetyltransferase (*pta*)-deficient mutant. As expected, inactivation of *pta* in the *als*-deficient mutant decreased the acetate level (Table 1). However, the metabolic defect of the *als* mutation was not recovered (Fig. 2; Table 1). These results indicate that the metabolic defects in the *als*-negative mutant are not directly caused by the observed increase in the acetate level.

Effect of expression of the *Z. mobilis pdc* and *aldB* genes in the *als*-deficient *K. pneumoniae* mutant

To evaluate whether the metabolic defects in the *als*-deficient mutant were related to accumulation of the metabolic intermediate, pyruvate, we expressed the pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (AldB) genes from *Z. mobilis* in the mutant strain to stimulate conversion of pyruvate into ethanol (Fig. 1). Following this genetic engineering, we detected higher activities of Pdc and AldB in the recombinant strain compared to the parent strain (Fig. 3), indicating that our genetic engineering was successful.

As shown in Fig. 2, heterologous expression of the *pdc* and *aldB* genes recovered the glycerol metabolism and cell growth of the *als*-negative mutant to levels similar to that of

Table 1 Metabolites of flask-cultivated *K. pneumoniae* mutant strains

	Δ*ldhA*	Δ(*ldhA als*)	Δ(*ldhA als pta*)	Δ(*ldhA als*)/pBR-*aldB-pdc*
Glycerol consumed (g L^{-1})	44.65	19.48	19.82	40.96
Cell growth (OD_{600})	3.67	2.30	2.52	3.48
1,3-PD (g L^{-1})	17.46	9.05	9.38	14.26
2,3-BD (g L^{-1})	5.38	0	0	0.12
Lactate (g L^{-1})	0	0	0	0
Succinate (g L^{-1})	1.75	0.74	1.08	1.44
Acetate (g L^{-1})	1.34	3.55	1.85	1.93
Ethanol (g L^{-1})	5.00	0.55	1.13	8.21

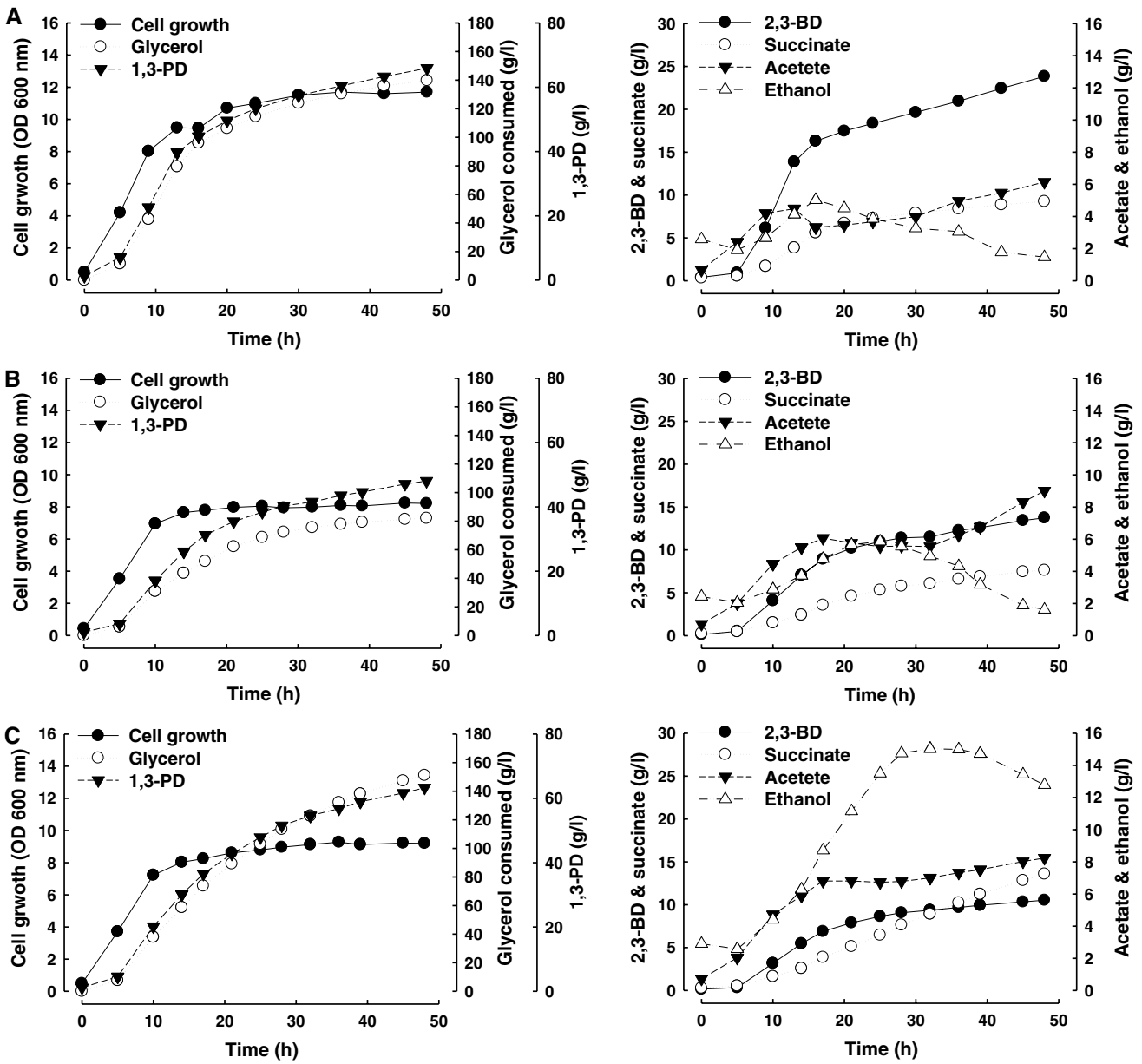

Fig. 4 Fermentation of *K. pneumoniae* mutant strains fed with crude biodiesel-industry-derived glycerol at an aeration rate of 2 vvm. **a** *K. pneumoniae* Δ*ldhA* mutant, **b** *K. pneumoniae* Δ(*ldhA als*) mutant, and **c** *K. pneumoniae* Δ(*ldhA als*) harboring pBR-*aldB-pdc*

the corresponding *als*-positive strain (Δ*ldhA*). The production of 1,3-PD was enhanced in the engineered strain, whereas 2,3-BD production remained entirely blocked (Table 1). Ethanol production was strikingly accelerated in the engineered strain, whereas acetate production was inhibited due to the stimulation of conversion of pyruvate to ethanol.

Fed-batch fermentation of the pBR-aldB-pdc-transfected Δ(*ldhA als*) mutant in a bioreactor

Following fed-batch fermentation, we observed similar 1,3-PD production levels in the *als*-positive Δ*ldhA* mutant and the Δ(*ldhA als*)/pBR-*aldB-pdc* mutant (Fig. 4; Table 2). The 2,3-BD level was lower in the Δ(*ldhA als*) mutant, as was the molar ratio of 2,3-BD to 1,3-PD. In contrast to our earlier batch-type cultivation, where 2,3-BD was not detected, we did detect 2,3-BD at different levels in the fed-batch-grown mutants.

A large amount of acetate accumulated in the culture broth of the *als*-negative mutant during fed-batch fermentation, due to the decreased conversion of pyruvate to 2,3-BD (Fig. 5). Notably however, the accumulation of acetate was completely complemented by expression of the *pdc* and *aldB* genes (Fig. 4; Table 2). This finding strongly suggests

Table 2 Metabolites of fed-batch-fermented *K. pneumoniae* mutant strains

	Δ*ldhA* (2.0 vvm)	Δ(*ldhA als*) (2.0 vvm)	Δ(*ldhA als*)/pBR-*aldB-pdc*		
			(0.5 vvm)	(2.0 vvm)	(3.0 vvm)
1,3-PD (g L^{-1})	65.9	48.0	49.4	63.3	68.2
2,3-BD (g L^{-1})	23.8	13.7	7.6	10.5	12.0
Lactate (g L^{-1})	0	0	0	0	0
Succinate (g L^{-1})	9.2	7.6	6.7	13.6	8.1
Acetate (g L^{-1})	6.1	9.0	7.0	8.2	16.7
Ethanol (g L^{-1})	5.0	5.9	20.2	15.0	8.0
1,3-PD yield (mol mol^{-1})	0.57	0.71	0.52	0.51	0.63
2,3-BD/1,3-PD (mol mol^{-1})	0.30	0.24	0.13	0.14	0.15
1,3-PD productivity (g L^{-1} h^{-1})	1.37	1.00	1.03	1.32	1.42

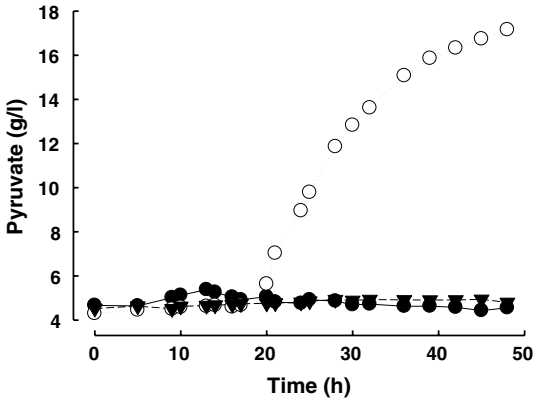

Fig. 5 Pyruvate analysis following fed-batch fermentation of the *K. pneumoniae* mutant strains. Symbols: *closed circles*, *K. pneumoniae* Δ*ldhA* harboring pBR322; *open circles*, *K. pneumoniae* Δ(*ldhA als*) harboring pBR322; *closed triangles*, *K. pneumoniae* Δ(*ldhA als*) harboring pBR-*aldB-pdc*

that the metabolic defects in the *als*-negative mutant are caused by accumulation of the metabolic intermediate, pyruvate, and may be recovered by metabolic engineering aimed at removing this accumulation (e.g., via the expression of *pdc* and *aldB*).

Effect of aeration rate on fed-batch fermentation of the Δ(*ldhA als*)-transfected pBR-*aldB-pdc* mutant

Finally, we evaluated the effect of different aeration rates on glycerol metabolism by the Δ(*ldhA als*)/pBR-*aldB-pdc* strain (Fig. 6). When the aeration rate was increased to 3.0 vvm from 2.0 vvm, the production level of 1,3-PD was slightly increased (Figs. 4c, 6b). The productivity and conversion rate were also enhanced by the fermentation condition, from 1.32 to 1.42 (g L^{-1} h^{-1}) and from 0.51 to 0.63 (mol mol^{-1}), respectively (Table 2). However, production of by-product 2,3-BD was also slightly increased. Contrastively, when aeration rate was reduced, 1,3-PD production significantly decreased. The decrease of 1,3-PD production might be due to the reduced cell growth. Low aeration rate also increased the ethanol level, further accompanying with the decrease of acetate level. Collectively, our results indicate that optimization of fermentation parameters together with the further metabolic engineering, such as the disruption of other *als* homologs, could further elevate 1,3-PD production in the *als*-negative mutant.

Discussion

The by-products of the fermentation, such as ethanol and acetate can be easily separated from 1,3-PD. However, 2,3-BD is a major by-product during the synthesis of 1,3-PD and it may serve as an obstacle for obtaining a high purity of 1,3-PD in downstream processes because of its similar boiling point [15].

In this study, we successfully used genetic engineering to enhance the production of 1,3-PD in the *K. pneumoniae* from glycerol while minimizing 2,3-BD production. We hypothesized that glycerol metabolism in the *als*-deficient mutant was affected by accumulation of the intermediate metabolite, pyruvate, and showed that heterologous expression of the *Z. mobilis pdc* and *aldB* genes efficiently recovered the metabolic defect and improved 1,3-PD production with cell growth in the *als*-deficient mutant by converting pyruvate to ethanol. However, the 1,3-PD production levels on glycerol is still slightly lower than Δ*ldhA* mutant and production of by-product ethanol was strikingly increased from 0.55 to 8.21 (g L^{-1}) (Table 1). This indicated that most of the NADH was consumed for ethanol production than 2,3-BD pathway and thus reduced the 1,3-PD production levels [19, 20]. These results may prove to be a promising alternative for enhancing the industrial production of 1,3-PD from crude glycerol while minimizing 2,3-BD production.

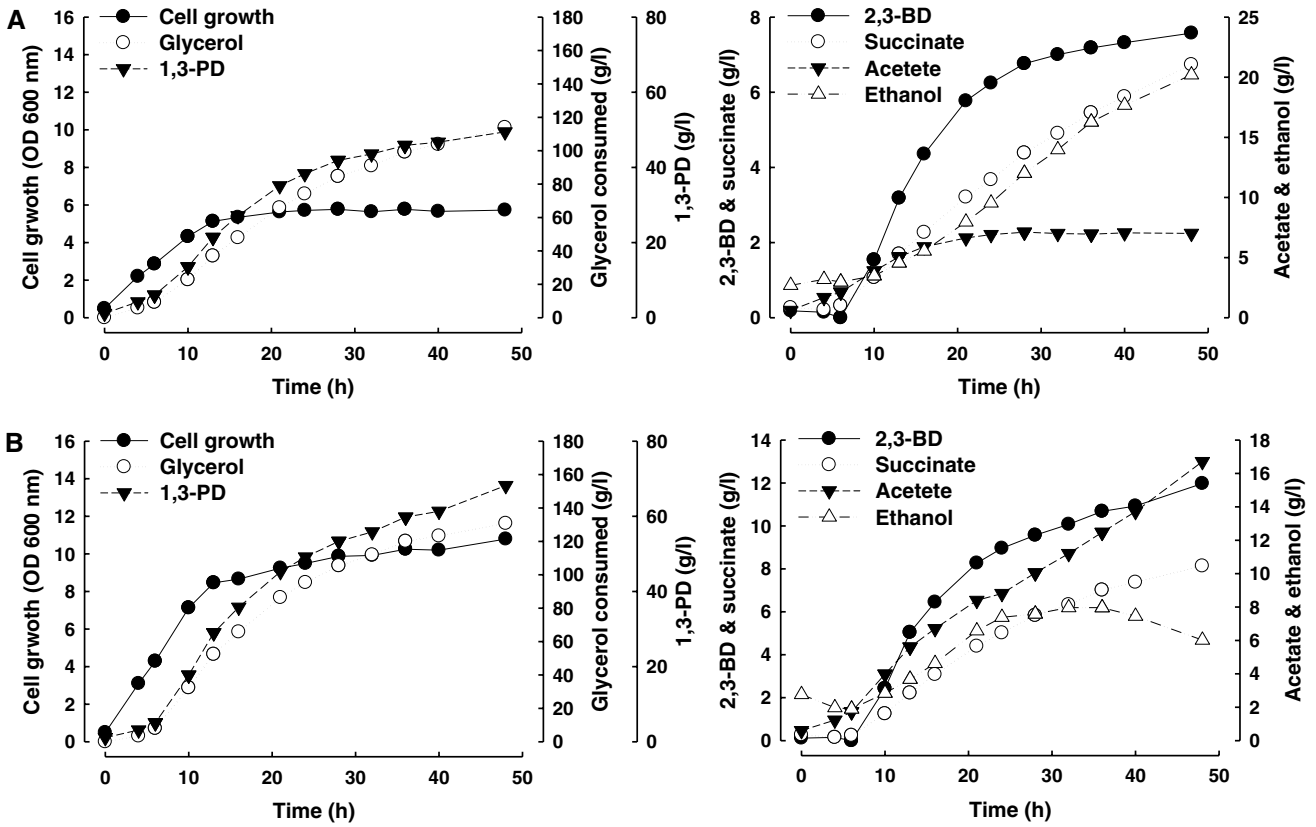

Fig. 6 Effect of aeration rate during fed-batch fermentation on the glycerol metabolism of *K. pneumoniae* Δ(*ldhA als*) harboring pBR-*aldB-pdc*. **a** 0.5 vvm, and **b** 3.0 vvm

We have previously identified and characterized an aldehyde/alcohol dehydrogenase (AdhE) involved in production of ethanol from glycerol in *K. pneumoniae* [21]. We also expressed *adhE* in the *als*-deficient mutant. However, 1,3-PD production was not recovered in the recombinant strain (data not shown). *K. pneumoniae* AdhE catalyzes synthesis of ethanol from acetyl-CoA not from pyruvate (Fig. 1). The results indicated that removal of accumulated pyruvate is crucial for the recovery of glycerol metabolism in Δ(*ldhA als*)/pBR-*aldB-pdc* strain.

In anaerobic and microaerobic conditions, acetate production is closely related with cell growth, which can supply ATP [22]. The cultivations carried out in this study were maintained at microaerobic condition irrespective of aeration rate. Agreed with, production of acetate was observed at growth stage, and then the production was stopped with the cessation of growth. The lower growth of Δ(*ldhA als*)/pBR-*aldB-pdc* strain and subsequent decreased production of 1,3-PD at lower aeration rate condition might be due to the stimulated production of ethanol from pyruvate, inhibiting acetate synthesis (Table 2). This means that precise optimization of cultivation condition is necessary for the best performance of engineered strain considering metabolic balance. Maximal production of 1,3-PD was observed at 3.0 vvm aeration rate by the Δ(*ldhA als*)/pBR-*aldB-pdc*. It has been also reported that growth of *K. pneumoniae* under microaerobic conditions (compared to anaerobic conditions) results in higher levels of 1,3-PD production [23].

In the *als*-deficient mutant of *K. pneumoniae*, 2,3-BD was still produced although the level was significantly decreased. It could be explained by presence of homologs of Als enzymes. Agreed with, genes annotated as acetolactate synthase I, II, and III were identified from the whole-genome sequences of *K. pneumoniae*. Differ to the *als*, the genes were not clustered with genes encoding acetolactate decarboxylase (Adc) and 2,3-BD dehydrogenase/acetoin reductase (Ard) involved in 2,3-BD biosynthesis. The gene products may be majorly involved in synthesis of branched chain amino acids such as valine in *K. pneumoniae,* in which, the biosynthetic pathway is branched out of acetolactate. The *als* homologs might be logical targets for further engineering in a future study.

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