

## RESEARCH ARTICLE

Impact of acetolactate synthase inactivation on 1,3-propanediol fermentation by *Klebsiella pneumoniae*Sheng Zhou<sup>1,2\*</sup>, Youhua Huang<sup>1,2</sup>, Xinliang Mao<sup>3</sup>, Lili Li<sup>2</sup>, Chuanyu Guo<sup>2</sup>, Yongli Gao<sup>2</sup>, Qiwei Qin<sup>1,2\*</sup>

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## Abstract

1,3-Propanediol (1,3-PDO) is an important compound that is mainly used in industry for polymer production. Fermentation of 1,3-PDO from glycerol by *Klebsiella pneumoniae* is accompanied by formation of 2,3-butanediol (2,3-BDO) as one of the main byproduct. The first step in the formation of 2,3-BDO from pyruvate is catalyzed by acetolactate synthase (ALS), an enzyme that competes with 1,3-PDO oxidoreductase for the cofactor NADH. This study aimed to analyze the impact of engineering the 2,3-BDO formation pathway via inactivation of ALS on 1,3-PDO fermentation by *K. pneumoniae* HSL4. An ALS mutant was generated using Red recombinase assisted gene replacement. The ALS specific activities of *K. pneumoniae*  $\Delta$ ALS were notably lower than that of the wild-type strain. Fed-batch fermentation of the mutant strain resulted in a 1,3-PDO concentration, productivity and conversion of 72.04 g L<sup>-1</sup>, 2.25 g L<sup>-1</sup> h<sup>-1</sup>, and 0.41 g g<sup>-1</sup>, increase by 4.71%, 4.65% and 1.99% compared with the parent strain. Moreover, inactivation of ALS decreased *meso*-2,3-BDO formation to trace amounts, significantly increased 2S,3S-BDO and lactate production, and a pronounced redistribution of intracellular metabolic flux was apparent.

## 1 Introduction

1,3-Propanediol (1,3-PDO) is an important industrial chemical intermediate that is used in the synthesis polymers [1, 2]. 1,3-PDO can be produced by both chemical synthesis and microbial fermentation. While chemical methods are troubled by poor selectivity and high energy consumption, microbial fermentation has a number of advantages including mild reaction conditions that are more environmentally favourable, greater sustainability, and easier operation [3, 4]. Numerous microorganisms produce 1,3-PDO including *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium butyricum*, and *Clostridium acetobutylicum* [5–7]. Of these, *K. pneumoniae* has been most widely explored since it achieves a higher concentration of 1,3-PDO, greater productivity, and a higher rate of conversion using glycerol as the carbon source [8, 9].

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In *K. pneumoniae* metabolism, glycerol is dissimilated through coupled reductive and oxidative pathways (Fig 1). 1,3-PDO is synthesized in the reductive pathway, whereas in the oxidative pathway, pyruvate-derived by-products such as 2,3-butanediol (2,3-BDO), acetate (AC), lactate (LAC), succinate (SUC) and ethanol (ETH) are generated through the channeling of glycerol into glycolysis [10]. Generation of by-products can compete with the biosynthesis of 1,3-PDO for carbon flow and NADH, which can lower the yield of 1,3-PDO. Blocking both the ETH and LAC pathways can reduce the production of ethanol and lactate and can increase the production of 1,3-PDO [11–13].

2,3-BDO is one of the the main byproducts generated alongside 1,3-PDO in the oxidative catabolism of glycerol, and both compounds share a similar high boiling point and water solubility. Therefore, 2,3-BDO not only competes for carbon flow, but also complicates 1,3-PDO recovery and purification [14]. Minimizing 2,3-BDO production would therefore improve the yield of 1,3-PDO and simplify the downstream processing [15, 16].

2,3-BDO is a metabolite derived from pyruvate (Fig 1). In the *Enterobacteriaceae* family, pyruvate from glycolysis can be converted into either LAC in an NADH-dependent reaction catalyzed by L-/D-LAC dehydrogenase (LDH) or, after decarboxylation, into acetolactate in a reaction catalyzed by acetolactate synthase (ALS), encoded by the *budB* gene. Acetolactate is mostly produced when NADH availability is low, and under aerobic conditions, it can be converted to acetoin by acetolactate decarboxylase (ALD), encoded by the *budA* gene. Under aerobic conditions, acetolactate can undergo spontaneous decarboxylation to produce diacetyl, and diacetyl reductase (DAR; also known as acetoin dehydrogenase) converts diacetyl to acetoin. Finally, butanediol dehydrogenase (BDH), encoded by the *budC* gene, also known as acetoin reductase, reduces acetoin to 2,3-BDO [17].

In this study, a 2,3-BDO pathway-deficient mutant of *K. pneumoniae* was constructed by knocking out the *budB* gene to generate a variant strain lacking ALS. The physiological and fermentation properties of the mutant strain, including the distribution and yield of various metabolites and products, were subsequently investigated. In particular, the influence that blocking the 2,3-BDO pathway has on glycerol metabolism and 1,3-PDO production was studied.

## 2 Materials and methods

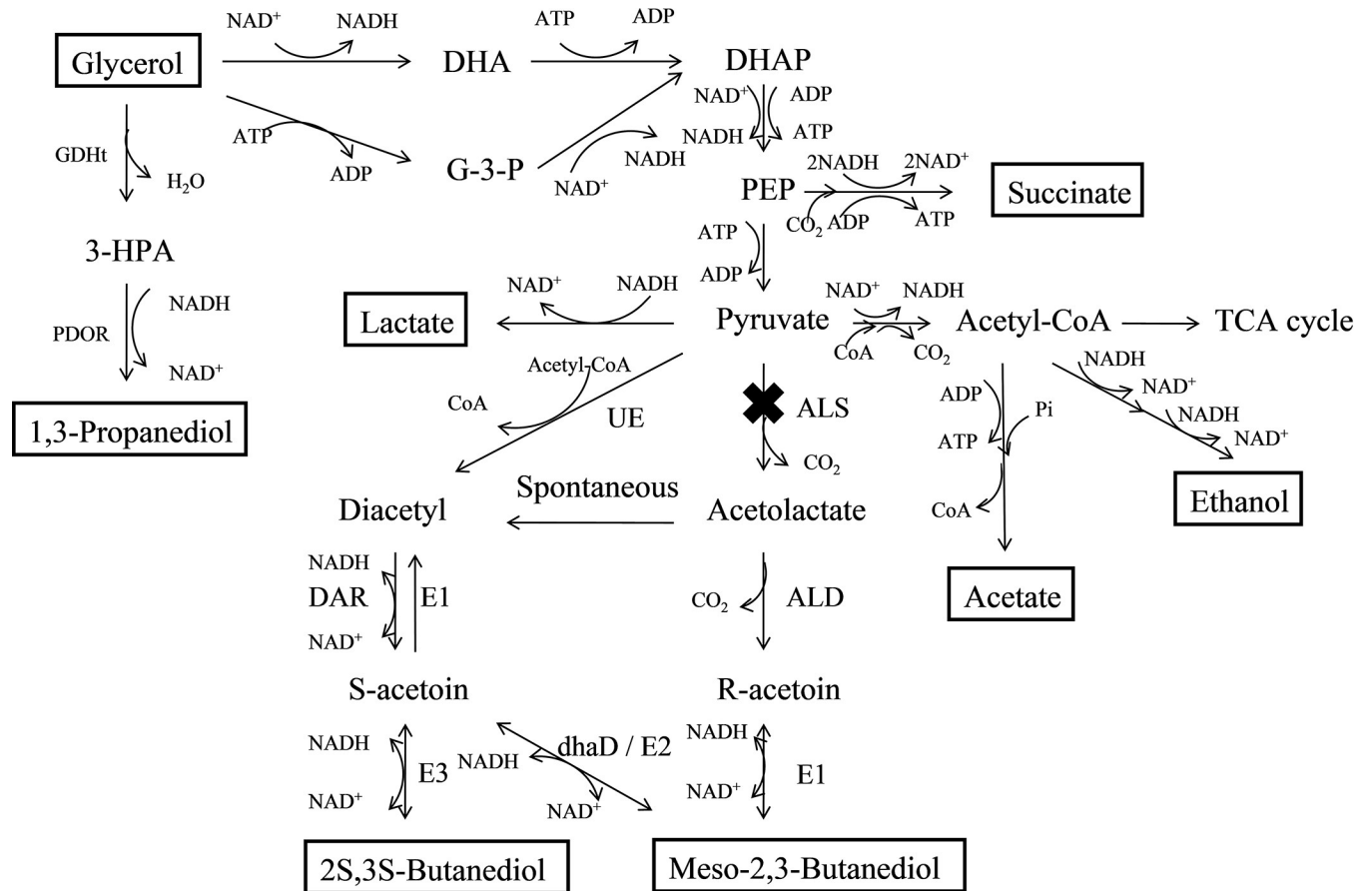
### 2.1 Strains, plasmids and culture media

Strains and plasmids used in this study are listed in Table 1. *Klebsiella pneumoniae* HSL4, used for the biosynthesis of 1,3-PDO in this work, was previously isolated from mangrove sediment samples [9]. Basic culture media contained the following components ( $L^{-1}$ ): 16 g pure glycerol (mass percent > 99%), 1.5 g yeast extract, 4 g  $(NH_4)_2SO_4$ , 0.69 g  $K_2HPO_4$ , 0.25 g  $KH_2PO_4$ , 0.2 g  $MgSO_4 \cdot 7H_2O$ , 1 ml trace element solution, and 1 ml  $Fe^{2+}$  solution. The trace element solution contained ( $L^{-1}$ ): 100 mg  $MgSO_4 \cdot 4H_2O$ , 70 mg  $ZnCl_2$ , 35 mg  $Na_2MoO_4 \cdot 2H_2O$ , 60 mg  $H_3BO_3$ , 200 mg  $CoCl_2 \cdot 6H_2O$ , 29.28 mg  $CuSO_4 \cdot 5H_2O$ , 25 mg  $NiCl_2 \cdot 6H_2O$ , and 0.9 ml HCl (37%).

When necessary, the medium was supplemented with antibiotics at the following final concentrations: 50  $\mu g mL^{-1}$  kanamycin (Kan), 100  $\mu g mL^{-1}$  ampicillin (Amp), 50  $\mu g mL^{-1}$  apramycin (Apra) or 25  $\mu g mL^{-1}$  chloramphenicol (Cm).

### 2.2 Construction of mutant strain *K. pneumoniae* $\Delta$ ALS

The ALS mutant strain *K. pneumoniae*  $\Delta$ ALS was constructed following a method described by Wei et al. [18]. Briefly, the ALS gene of *K. pneumoniae* HSL4 was amplified by PCR using primer pair ALS-F1/ALS-R1. The PCR product was ligated into the pMD18-T simple vector to



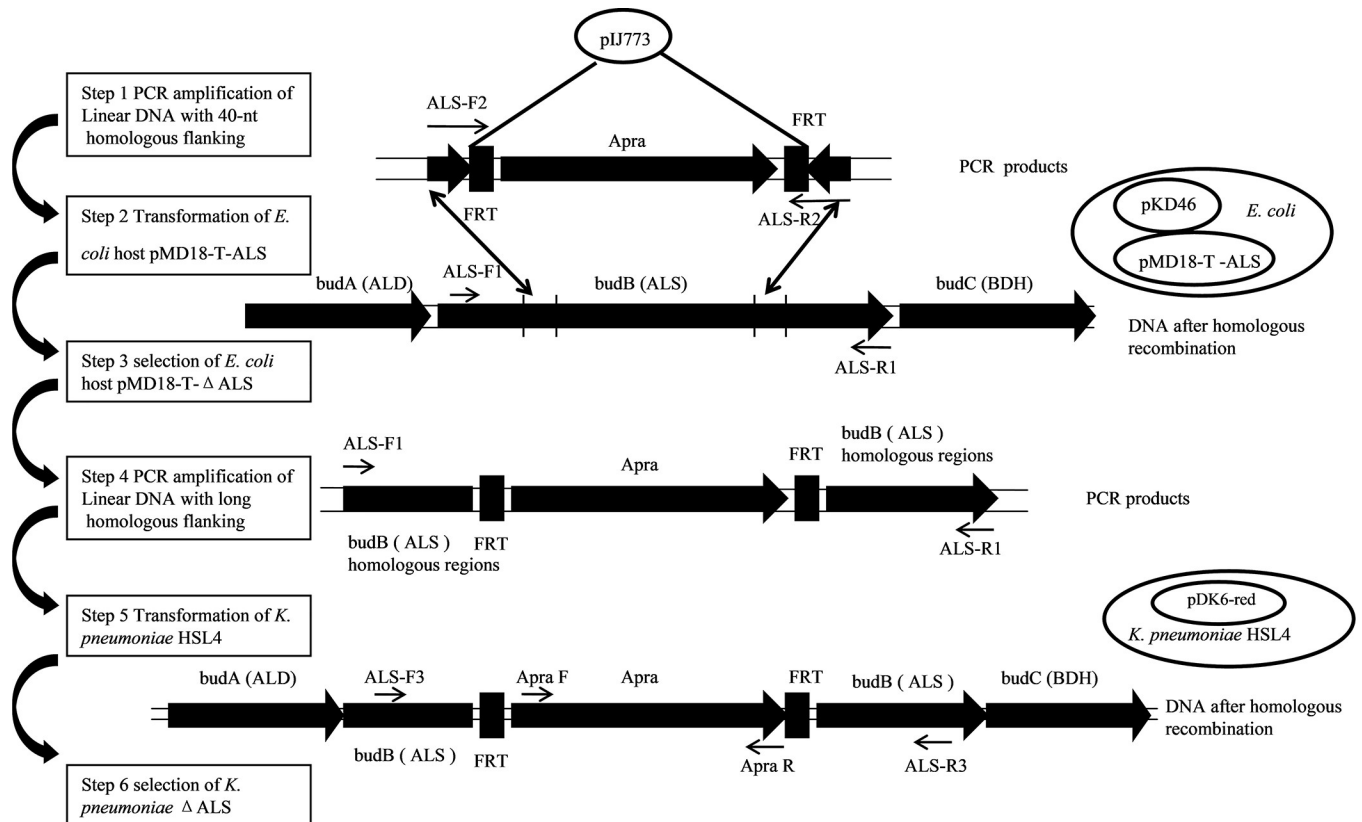
**Fig 1. Metabolic pathways of glycerol metabolism and the proposed mechanism for the biosynthesis of 2,3-BDO stereoisomers in *K. pneumoniae*.** The glycerol substrate and major product metabolites are boxed. UE: Unknown enzyme; ALS: acetolactate synthase (mutated in this study); ALD:  $\alpha$ -acetolactate decarboxylase; DAR: diacetyl reductase; E1: *meso*-2,3-BDO dehydrogenase (R-acetoin forming); E2: *meso*-2,3-BDO dehydrogenase (S-acetoin forming); E3: (S, S)-2,3-BDO dehydrogenase; dhaD: Glycerol dehydrogenase; DHA: Dihydroxyacetone, DHAP: Dihydroxyacetone phosphate; G-3-P: glycerol-3-Phosphate; PEP: phosphoenolpyruvate; GDHt: glycerol dehydratase; PDOR: 1,3-propanediol oxidoreductase; 3-HPA: 3-hydroxypropionaldehyde; TCA: tricarboxylic acid cycle.

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**Table 1. Bacterial strains and plasmids.**

Strain or plasmid	Relevant genotype and description	Source or reference
Strains		
<i>K. pneumoniae</i> HSL4	Wild-type, CCTCC M 2011075	[9]
<i>K. pneumoniae</i> pDK6-red	<i>K. pneumoniae</i> CCTCC M2011075, pDK6-red	[18]
<i>K. pneumoniae</i> $\Delta$ ALS	<i>K. pneumoniae</i> CCTCC M2011075, $\Delta$ ALS, aac(3)IV	This work
<i>Escherichia coli</i> DH5a	Host of plasmid	Lab stock
Plasmids		
pMD18-T simple	Amp <sup>r</sup> , TA cloning vector, 2692 bp	TaKaRa
pMD18-T-ALS	Amp <sup>r</sup> , carries ALS, 4231 bp	This work
pMD18-T- $\Delta$ ALS	Amp <sup>r</sup> , carries $\Delta$ ALS, 5230 bp	This work
pDK6-red	Kan <sup>r</sup> , carries $\lambda$ -Red genes (gam, bet, exo), 7.1 kb	[18]
pDK6	Kan <sup>r</sup> , lacIQ, tac, 5.1 kb	[19]
pKD46	Amp <sup>r</sup> , $\lambda$ Red recombinase expression	[20]
pIJ773	Apra <sup>r</sup> , aac(3)IV with FRT sites, 4334 bp	[21]

<https://doi.org/10.1371/journal.pone.0200978.t001>



**Fig 2. Methodology for ALS gene mutation using Red recombination.** BudA (ALD) and budC (BDH) are upstream and downstream of ALS on the *K. pneumoniae* HSL4 chromosome, respectively. ALS-F1/R1, ALS-F2/R2, ALS-F3/R3, and Apra F/R were the primer pairs used.

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generate pMD18-T-ALS. A linear portion of DNA with both 40 nt homologous extensions flanking the apramycin resistance gene *aac(3)IV* was amplified using plasmid pIJ773 as the template with primer pair ALS-F2/ALS-R2. The pMD18-T-ΔALS construct was engineered by replacing part of the ALS gene in plasmid pMD18-T-ALS with the *aac(3)IV* cassette using the Red/ET system in *Escherichia coli* [20]. The pMD18-T-ΔALS vector was used as a template for the PCR-based preparation of a linear DNA containing the apramycin resistance gene *aac(3)IV* with 468 bp upstream and 714 bp downstream homologous regions (long homologous flanking). Finally, the long homologous flanking linear DNA product was transformed into *K. pneumoniae*-pDK6-red, which already hosted the pDK6-red plasmid. Homologous recombination between the introduced linear DNA and chromosomal DNA was facilitated by Red recombinase and led to the deletion of ALS (Fig 2). The ALS-deficient strains were selected on apramycin plates, and primer pairs Apra-F/Apra-R and ALS-F3/ALS-R3 were used for PCR confirmation. Primers used in this study are listed in Table 2.

### 2.3 Batch and fed-batch fermentation

For batch fermentation and preparation of seed cultures for fed-batch fermentation, 250 mL flasks containing 100 mL of basic culture medium (glycerol adjusted to 30 g L<sup>-1</sup>) were inoculated with a single colony and incubated at 37°C in a rotatory shaker at 200 rpm for 12 h. For fed-batch fermentation, a 3% (v/v) inoculum was added aseptically to a 5 L fermenter (Zheng Jiang Dong Fang, China) containing 3 L of media. NaOH (8 M) was used to maintain the pH. Aerobic fermentation according to Hao et al. [22] was carried out at 37°C, pH 6.8, 200 rpm

Table 2. Primers used in this study.

Primer name	Sequence (5'-3')
ALS-F1	ATGGACAAACAGTATCCGGTACGC
ALS-R1	TTACAGAATCTGACTCAGATGCAG
ALS-F2	CGCCGCGCCGGATGATGCCATCGACCAGGTGGCGAAGCTTTCCGGGGATCCGTCGACC
ALS-R2	CGATATTTTGCGCCAGCTTGTGAGAGTGCCGGCGATATCGTAGGCTGGAGCTGCTTC
ALS-F3	AAGCCAACGCCGCATTTATGGC
ALS-R3	CATCAGCAGCGGGTTATCGCGAT
Apra-F	TTCCGGGGATCCGTCGACC
Apra-R	GTAGGCTGGAGCTGCTTC

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with an air flow of 0.5 vvm (volume per volume per minute). The glycerol concentration was 16–18 g L<sup>-1</sup> in the culture medium at the beginning of fermentation, and the consumption of glycerol was slow during fermentation for 0–5 h, when the glycerol concentration dropped below 5 g L<sup>-1</sup>, additional pure glycerol was added to the culture medium to maintain the concentration of glycerol was between 2 to 20 g L<sup>-1</sup> in subsequent fed-batch cultures at 5–32 h. Total amount of glycerol consumption was calculated after the fermentation was completed.

## 2.4 The ALS activity assays

The ALS assay was based on enzymatic conversion of pyruvate to acetolactate and acetolactate converts to acetoin by acid hydrolysis. The assay was conducted as described by Yang et al. [23]. Cell samples were collected every 1–4 h during the fermentation and centrifuged at 5000 × g, 4°C for 10 min and pellets were stored at 4°C until needed for analysis. Pellets were washed with cold 50 mM acetate buffer pH 6, centrifuged at 5000 × g, 4°C for 10 min, resuspended in the same buffer at 4°C, and crude cell extracts (for use in enzyme assays) were obtained by sonication with an ultrasonic cell disrupter. Reaction mixtures contained 20 mM sodium pyruvate, 50 mM acetate buffer pH 6, 1 mM MgCl<sub>2</sub>, and 80 mL of cocarboxylase. Reactions were initiated by the addition of 100 μL of crude extract to 1 mL of reaction buffer and followed for 20 min at 37°C. Reactions were terminated by addition of 0.1 mL of 50% H<sub>2</sub>SO<sub>4</sub>. Mixtures were incubated for an additional 25 min at 37°C to allow acid hydrolysis of acetolactate to acetoin, and acetoin was measured using the Voges–Proskauer test. Briefly, a 0.2 mL aliquot of the aforementioned mixture was transferred to a tube with 0.8 mL of 0.45 M NaOH, and 1 mL of α-naphthol (5% in 2.5 M NaOH) and 0.5% creatine (1:1) was added. Reactions were incubated at room temperature for 30 min with slightly shaking, and the absorbance at 535 nm was measured. Calibration with pure acetoin showed a correlation of 1 OD<sub>535</sub> unit to 0.15 mM acetoin. One specific unit of ALS activity corresponds to the formation of 1 mmol of acetoin per mg of protein per minute. All the data were expressed as means from three biological replicate experiments.

## 2.5 Biomass and fermentation broth analysis

Cell density was monitored using a spectrophotometer at 600 nm (OD<sub>600nm</sub>). Cell density was expressed as OD<sub>600nm</sub> or by converting OD<sub>600nm</sub> to biomass (g L<sup>-1</sup>) according to the curve set up previously in our laboratory. Glycerol, 1,3-PDO, 2,3-BDO, LAC, SUC, AC, ETH, 2S,3S-BDO and meso-2,3-BDO were determined using a Shimadzu 20AT HPLC system with an RID-10A refractive index detector and equipped with a 300 × 7.8mm<sup>2</sup> Aminex HPX-87H column (Bio-Rad, USA) running at 0.8 ml min<sup>-1</sup> after adding 5 mM H<sub>2</sub>SO<sub>4</sub>. The column

temperature was controlled at 65°C. All the data were expressed as means from three biological replicate experiments.

### 3 Results

#### 3.1 Inactivation of the ALS gene in *K. pneumoniae* HSL4

A Red recombination approach was used to knock-out the ALS gene in *K. pneumoniae* HSL4 (Fig 2). A positive recombinant was selected from the apramycin plate, confirmed by PCR and named *K. pneumoniae*  $\Delta$ ALS.

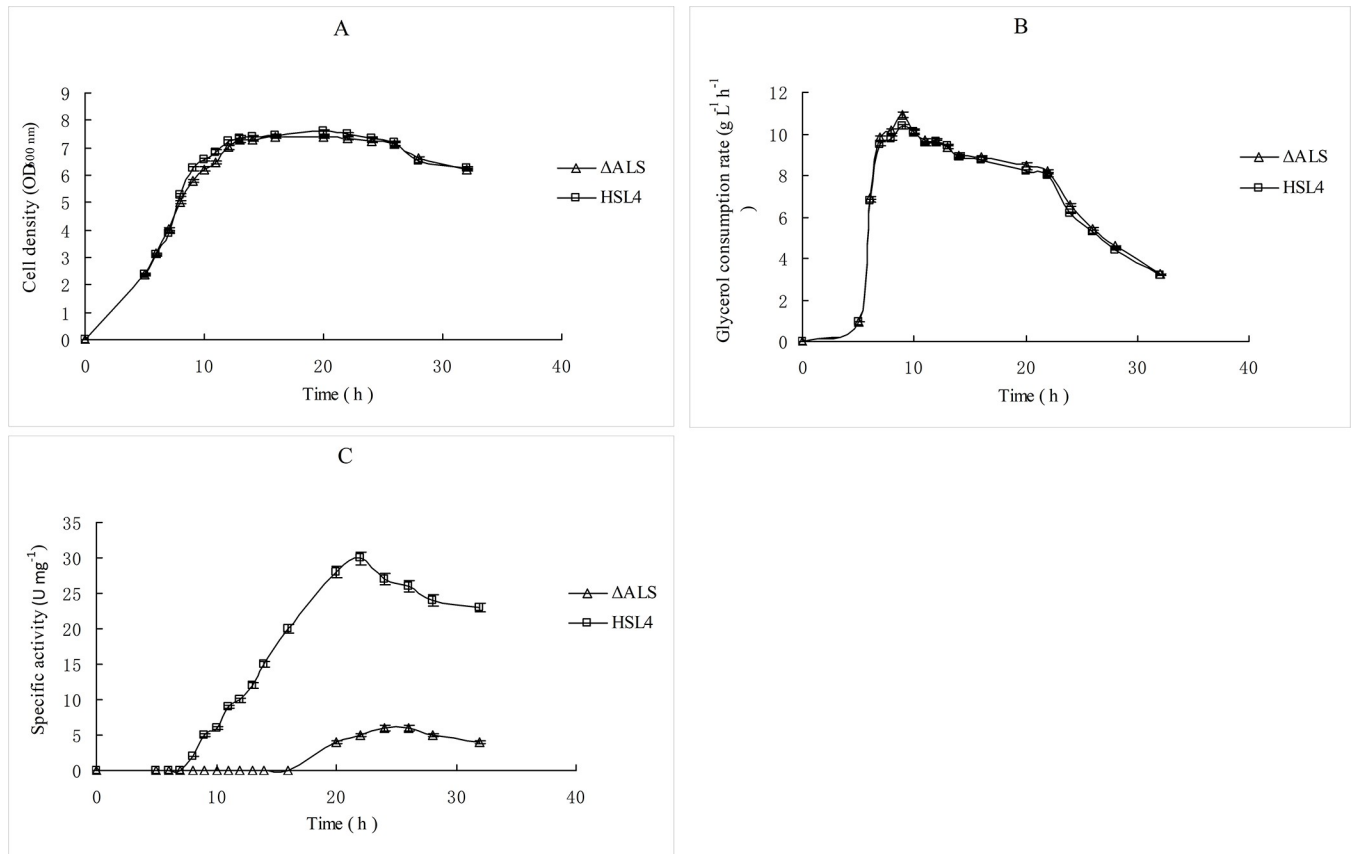
#### 3.2 Effect of ALS inactivation on cell growth, glycerol consumption and ALS enzymatic activity

The effect of ALS inactivation on cell growth was examined during fermentation (Fig 3A). The maximum OD<sub>600nm</sub> (biomass) reached was 6.80, and the growth of *K. pneumoniae*  $\Delta$ ALS was unaffected compared with the wild-type strain. Consumption of glycerol by *K. pneumoniae*  $\Delta$ ALS and *K. pneumoniae* HSL4 were also monitored in a fed-batch culture, and the glycerol consumption rate (5.50 g L<sup>-1</sup> h<sup>-1</sup>) of *K. pneumoniae*  $\Delta$ ALS was slightly higher than that of *K. pneumoniae* HSL4 (5.34 g L<sup>-1</sup> h<sup>-1</sup>), as shown in Fig 3B. Further investigation revealed a notably lower ALS specific activity in the crude cell extract of *K. pneumoniae*  $\Delta$ ALS than the wild-type strain (Fig 3C). These results indicated that the consumption of glycerol was slightly promoted by inactivation of ALS.

#### 3.3 Fed-batch fermentation of glycerol to 1,3-PDO by *K. pneumoniae* $\Delta$ ALS

Fed-batch cultures were carried out in a 5 L fermenter to further investigate the fermentation of glycerol to 1,3-PDO by *K. pneumoniae*  $\Delta$ ALS, and the results after 32 h are shown in Table 3 and Fig 4. After 5–16 h, the substrate was consumed fast and 1,3-PDO was rapidly synthesized by both *K. pneumoniae*  $\Delta$ ALS and the wild-type strain. However between 16–26 h, substrate uptake and the 1,3-PDO synthesis were slower, and both LAC and *meso*-2,3-BDO were rapidly accumulated by *K. pneumoniae* HSL4, while only LAC was rapidly accumulated by *K. pneumoniae*  $\Delta$ ALS. After 32 h, 1,3-PDO was the main product and reached a concentration of 72.04 g L<sup>-1</sup> in the broth by *K. pneumoniae*  $\Delta$ ALS and 68.76 g L<sup>-1</sup> in the broth of the wild-type strain. Fermentation by *K. pneumoniae*  $\Delta$ ALS produced five other main metabolites including 31.50 g L<sup>-1</sup> LAC, 8.30 g L<sup>-1</sup> SUC, 9.60 g L<sup>-1</sup> AC, 7.60 g L<sup>-1</sup> ETH, and 2.16 g L<sup>-1</sup> 2S,3S-BDO. In comparison, *K. pneumoniae* HSL4 produced 15.42 g L<sup>-1</sup> LAC, 7.76 g L<sup>-1</sup> SUC, 7.02 g L<sup>-1</sup> AC, 9.23 g L<sup>-1</sup> ETH, and 13.75 g L<sup>-1</sup> *meso*-2,3-BDO.

As shown in Table 3, glycerol consumption by *K. pneumoniae*  $\Delta$ ALS was slightly higher than that of the parent strain. 1,3-PDO reached a final concentration of 72.04 g L<sup>-1</sup> in *K. pneumoniae*  $\Delta$ ALS, which was a slightly increase of 4.71% compared to the parent strain. 1,3-PDO productivity was 2.25 g L<sup>-1</sup>h<sup>-1</sup> with *K. pneumoniae*  $\Delta$ ALS, higher than *K. pneumoniae* HSL4 (2.15 g L<sup>-1</sup>h<sup>-1</sup>). Similarly, there was a slightly increase in 1,3-PDO conversion of *K. pneumoniae*  $\Delta$ ALS comparing to the parent strain. Therefore, as expected, ALS inactivation decreased *meso*-2,3-BDO generation, although *K. pneumoniae*  $\Delta$ ALS produced increased LAC (31.50 g L<sup>-1</sup>) than the parent strain (15.42 g L<sup>-1</sup>). This suggests that ALS deletion shifted the carbon flux away from the biosynthesis of *meso*-2,3-BDO towards the production of LAC and made 1,3-PDO production, productivity and conversion (yield) slightly increased but none of these parameters were changed significantly.



**Fig 3.** Time course of cell growth (OD<sub>600nm</sub>) (A), glycerol consumption rate (B), and ALS specific activity (C) of *K. pneumoniae*  $\Delta$ ALS and *K. pneumoniae* HSL4 in fed-batch culture. Enzyme activities are expressed in mM mg<sup>-1</sup> min<sup>-1</sup> (U mg<sup>-1</sup>). Data were expressed as means from three biological replicate experiments.

<https://doi.org/10.1371/journal.pone.0200978.g003>

### 3.4 Metabolic profiling of fed-batch fermentation by *K. pneumoniae* $\Delta$ ALS

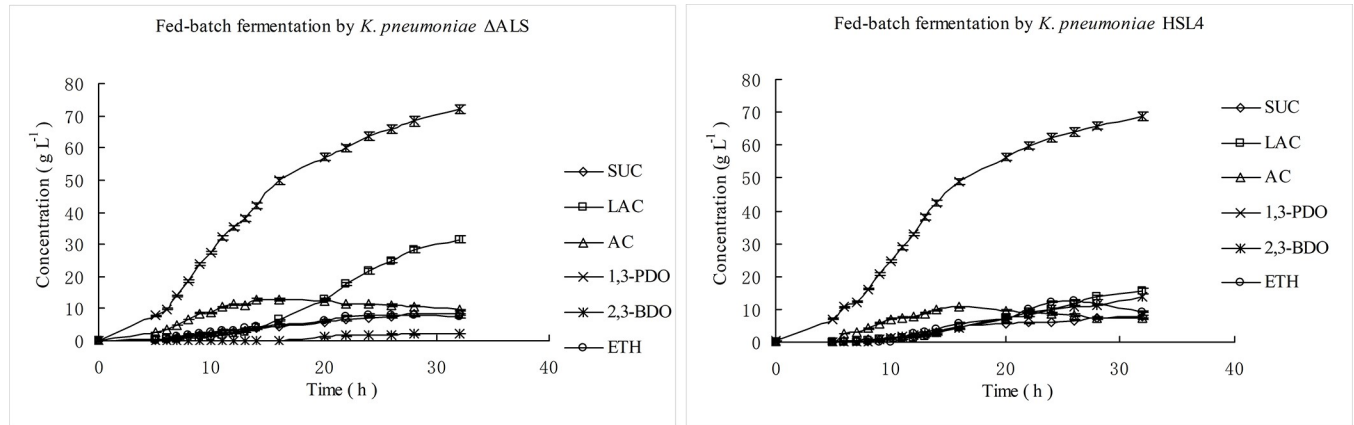
Based on the results of cell growth, substrate consumption and metabolite formation profiles (Figs 3–4), fed-batch fermentations were divided into four periods; (I) Initial phase (0–5 h), (II) exponential growth phase (5–16 h), (III) stationary phase (16–26 h) and (IV) decline phase (26–32 h). Metabolic flux during different phases of the *K. pneumoniae*  $\Delta$ ALS and HSL4 fed-batch fermentations showed that during period I, the cell biomass was low but increase rapidly (Fig 3A and Table 4). In this period, cells soon adapted to the new culture conditions, and the rate of substrate consumption and 1,3-PDO formation was high. Unfortunately, only a small amount of 1,3-PDO was synthesized due to the low biomass. During period II, cells grew

**Table 3.** Fed-batch fermentation of glycerol by *K. pneumoniae*  $\Delta$ ALS and *K. pneumoniae* HSL4 after 32 h.

Strains	Substrate consumption (g L <sup>-1</sup> )	Metabolites (g L <sup>-1</sup> )						1,3-PDO productivity (g L <sup>-1</sup> h <sup>-1</sup> )	1,3-PDO conversion (g g <sup>-1</sup> )
	Glycerol	1,3-PDO	LAC	SUC	AC	2,3-BDO	ETH		
$\Delta$ ALS	175.91 ± 2.32	72.04 ± 1.46	31.50 ± 0.78	8.30 ± 0.28	9.60 ± 0.26	2.16 ± 0.15	7.60 ± 0.32	2.25 ± 0.03	0.410 ± 0.008
HSL4	170.90 ± 2.24	68.76 ± 1.33	15.42 ± 0.89	7.76 ± 0.25	7.22 ± 0.21	13.75 ± 0.22	9.23 ± 0.19	2.15 ± 0.03	0.402 ± 0.007

Averages ± standard deviation were deduced from three biological replicate experiments. 1,3-PDO, 1,3-propanediol; LAC, Lactate; SUC, Succinate; ACE, Acetate; 2,3-BDO, 2,3-Butanediol; ETH, Ethanol.

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**Fig 4. Time course of fed-batch fermentation of glycerol to 1,3-propanediol by *K. pneumoniae* ΔALS and *K. pneumoniae* HSL4 under micro-aerobic conditions.** 1,3-PDO, 1,3-propanediol; LAC, Lactate; SUC, Succinate; ACE, Acetate; 2,3-BDO, 2,3-Butanediol; ETH, Ethanol. Data were expressed as means from three biological replicate experiments.

<https://doi.org/10.1371/journal.pone.0200978.g004>

exponentially, and both biomass and 1,3-PDO concentration increased sharply. Other metabolites were also synthesized steadily, but 1,3-PDO was the main product during this period. Acetate increased rapidly during the exponential growth period (5–16 h) and carbon flux was diverted to LAC synthesis in the stationary growth period (16–26 h), whereas 1,3-PDO synthesis slowed down during this phase. During periods III and IV, large amounts of LAC accumulated in *K. pneumoniae* ΔALS. Period IV is known as the decline phase, during which cell growth ceases, cells begin to die, and substrate uptake and metabolite formation decrease, although LAC synthesis continued in both *K. pneumoniae* ΔALS and *K. pneumoniae* HSL4. Other metabolites exhibited a similar flux distribution, although ethanol and acetate synthesis was negative due to volatilization and reutilization, respectively.

The distribution of metabolites in fed-batch fermentations by *K. pneumoniae* ΔALS and *K. pneumoniae* HSL4 (Table 4) clearly showed that the flow of glycerol to 1,3-PDO and byproducts other than *meso*-2,3-BDO and ETH was increased in the mutant *K. pneumoniae* ΔALS compared with the parent strain. It is worth noting that LAC increased strikingly in *K. pneumoniae* ΔALS, and it was reported that *meso*-2,3-BDO increased notably in a LAC-deficient mutant [12, 13]. In glycerol metabolism, LAC and *meso*-2,3-BDO reside on separate branches

**Table 4. Metabolic flux in fed-batch fermentation by *K. pneumoniae* ΔALS and *K. pneumoniae* HSL4.**

Strains	Fermentation period	Biomass (g L <sup>-1</sup> )	Glycerol consumption (m mol g <sup>-1</sup> h <sup>-1</sup> )	Metabolites (m mol g <sup>-1</sup> h <sup>-1</sup> )					
				1,3-PDO	LAC	SUC	AC	2,3-BDO	ETH
ΔALS	I(0<t<5 h)	2.40 ± 0.03	12.68 ± 0.30	8.50 ± 0.22	0.00	0.00	0.00	0.00	0.00
HSL4	I(0<t<5 h)	2.37 ± 0.05	13.20 ± 0.40	7.83 ± 0.21	0.00	0.00	0.00	0.00	0.00
ΔALS	II(5<t<16 h)	9.80 ± 0.60	8.62 ± 0.35	5.13 ± 0.23	0.63 ± 0.02	0.32 ± 0.02	1.57 ± 0.03	0.00	0.99 ± 0.04
HSL4	II(5<t<16 h)	9.82 ± 0.63	8.43 ± 0.32	5.10 ± 0.20	0.43 ± 0.01	0.36 ± 0.03	1.65 ± 0.04	0.46 ± 0.02	1.15 ± 0.05
ΔALS	III(16<t<26 h)	14.53 ± 0.24	4.35 ± 0.36	1.47 ± 0.15	1.41 ± 0.08	0.18 ± 0.01	0.21 ± 0.01	0.14 ± 0.01	0.45 ± 0.02
HSL4	III(16<t<26 h)	14.63 ± 0.19	4.23 ± 0.33	1.36 ± 0.17	0.58 ± 0.05	0.11 ± 0.01	-0.28 ± 0.01	0.48 ± 0.02	1.03 ± 0.04
ΔALS	IV(26<t<32 h)	13.33 ± 0.20	2.47 ± 0.15	0.99 ± 0.05	0.92 ± 0.03	0.08 ± 0.01	-0.29 ± 0.01	0.04 ± 0.01	-0.08 ± 0.01
HSL4	IV(26<t<32 h)	13.43 ± 0.16	2.14 ± 0.14	0.74 ± 0.04	0.51 ± 0.02	0.13 ± 0.01	-0.20 ± 0.01	0.41 ± 0.02	-0.93 ± 0.04

Average ± standard deviation, were deduced from three biological replicate experiments. 1,3-PDO, 1,3-propanediol; LAC, Lactate; SUC, Succinate; ACE, Acetate; 2,3-BDO, 2,3-Butanediol; ETH, Ethanol.

<https://doi.org/10.1371/journal.pone.0200978.t004>



downstream from pyruvate and both depend on the action of dehydrogenase enzymes that use NADH as a cofactor. In general, one pathway is likely to be strengthened when the other is blocked, therefore it is logical that blocking *meso*-2,3-BDO biosynthesis resulted in the promotion of LAC biosynthesis in this study.

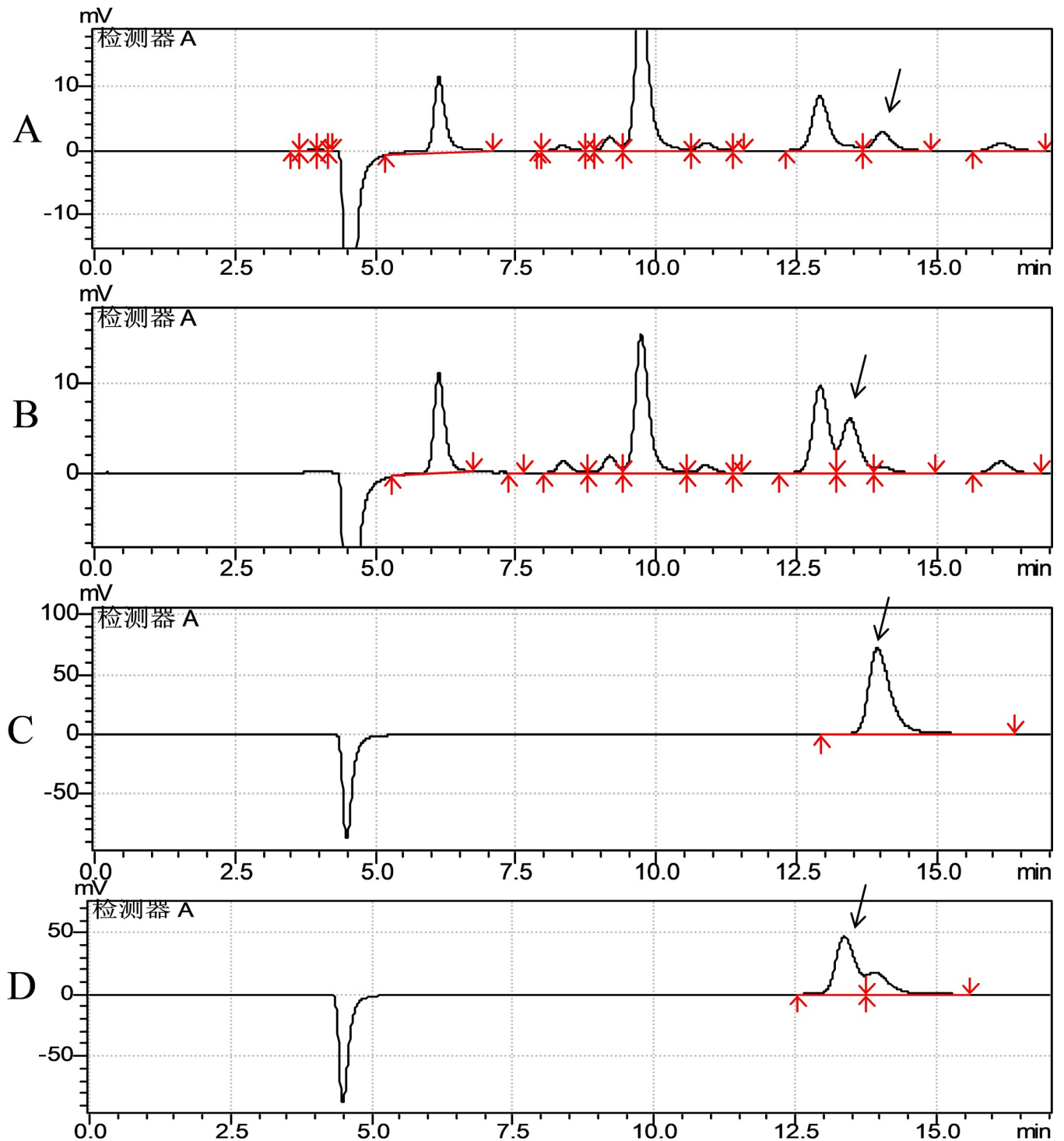
### 3.5 2,3-BDO stereoisomers in *K. pneumoniae* $\Delta$ ALS and *K. pneumoniae* HSL4

*Klebsiella pneumoniae* synthesizes both *meso*-2,3-BDO and 2S,3S-BDO through fermentation of glycerol [17]. In this study, both mutant and the wild-type strains were cultured with glycerol (30 g L<sup>-1</sup>) as the carbon source, and 1,3-PDO was the main product with both strains (Fig 5). However, the strains produced different amounts of 2,3-BDO of isomers; wild-type *K. pneumoniae* HSL4 produced 3.8 g L<sup>-1</sup> *meso*-2,3-BDO and 0.20 g L<sup>-1</sup> 2S,3S-BDO after fermentation for 12 h, whereas *K. pneumoniae*  $\Delta$ ALS produced 0.16 g L<sup>-1</sup> and 0.85 g L<sup>-1</sup>, respectively. A distinguishing characteristic of the mutant strain was the low level of *meso*-2,3-BDO and high level of 2S,3S-BDO produced, suggesting synthesis of *meso*-2,3-BDO was inhibited while the formation of 2S,3S-BDO was stimulated in the absence of ALS.

## 4 Discussion

During growth on glycerol in *K. pneumoniae*, considerable amounts of metabolites are produced via the activities of oxidative branch enzymes [10]. Lactate, 2,3-BDO, ETH, SUC and AC are among the major by-product metabolites generated, and these can account for 40%–45% of the total metabolites. In this study, LAC (15.42 g L<sup>-1</sup>) and 2,3-BDO (13.75 g L<sup>-1</sup>) were the two most abundant by-products and reached 22.4% and 20.0% (w/w) that of 1,3-PDO (68.76 g L<sup>-1</sup>), which were the major products in the fermentation broth of the wild-type strain. Minimizing the production of by-products is an effective way to engineer the microbial strain to synthesize more 1,3-PDO. Both ETH [11] and LAC pathways of *K. pneumoniae* [13] and *Klebsiella oxytoca* [12] have been successfully disrupted by knocking out genes encoding key enzymes in these pathways. Inactivation of the ETH pathway by knocking-out ALDH in *K. pneumoniae* YMU2 almost abolished ETH formation and significantly increased 1,3-PDO production [11]. Similarly, Yang et al. [12] reported that LAC-deficient mutants of *K. oxytoca* were obtained by knocking out the gene encoding LDH. Their results showed that 1,3-PDO concentration, productivity, and conversion rate can be enhanced above levels achieved by wild-type strains, and LAC production can be diminished. However in their study, the distribution of metabolites was altered and over 60 g L<sup>-1</sup> 2,3-BDO was produced as the major byproduct. Previously, the D-LAC pathway of *K. pneumoniae* HR526 was disrupted by knocking out D-LAC dehydrogenase [13], which increased conversion of 1,3-PDO and 2,3-BDO from 0.55 mol mol<sup>-1</sup> to 0.65 mol mol<sup>-1</sup>, and the final LAC concentration decreased dramatically from more than 40 g L<sup>-1</sup> to less than 3 g L<sup>-1</sup>.

To improve both the yield and productivity of 1,3-PDO from glycerol, disruption of the 2,3-BDO pathway is an alternative strategy. In *K. pneumoniae*, the genes coding acetolactate decarboxylase (ALD, encoded by the *budA* gene), acetolactate synthase (ALS, encoded by the *budB* gene) and butanediol dehydrogenase (BDH, encoded by the *budC* gene) catalyse the synthesis of 2,3-BDO [17]. The results of previous studies revealed that mutation of ALD not only decreased carbon flux to 2,3-BDO, but also increased the ratio of NADH to NAD<sup>+</sup> by decreasing NADH consumption due to 2,3-BDO synthesis. Consequently, carbon flux was diverted towards the biosynthesis of 1,3-PDO [15]. Similarly, inactivation of BDH and expression of formate dehydrogenase reduced 2,3-BDO production and increased 1,3-PDO production [24], and disruption of the *budC* gene in *K. pneumoniae* ZG38 produced the same result [16].



**Fig 5.** HPLC analysis of *K. pneumoniae*  $\Delta$ ALS (A) and *K. pneumoniae* HSL4 (B) fermentation broth following growth on glycerol. The arrows pointing to the peaks in (A) and (B) indicate 2S,3S-BDO and *meso*-2,3-BDO, respectively. Standards of 2S,3S-BDO and *meso*-2,3-BDO were shown by arrows pointing to the peaks in (C) and (D), respectively. Peaks corresponding to other metabolites and substrates are not indicated.

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Cui et al. [25] reported an improvement in the intracellular redox state in 2,3-BDO-deficient mutants lacking *budA* and *B*. Additionally, excess NADH was produced in the LAC-deficient

mutant overexpressing *dhaT*. Since the synthesis of 2,3-BDO is catalysed procedurally by budB, A and C and taking into account the reports that the mutation of budA and C promoted 1,3-PDO production, it is deductive that budB disruption should also be beneficial to the 1,3-PDO production. However, Oh et al. [26] reported the deletion of ALS gene (budB), hampering efficient 1,3-PDO production. The logical prediction was contradicted with the results by Oh et al. [26]. That implied the function of ALS gene (budB) referring to glycerol metabolism was deserved to be further investigated. The results from this work were disagreed with the results by Oh et al. [26]. There may difference in fermentation condition between Oh et al. [26] and our study. In addition, the isomers of 2,3-BDO have been distinguished in our study but not in Oh et al. [26]

In this study, an ALS-deficient strain was constructed using the Red recombination system, and fermentation tests showed that inactivation of ALS caused a redistribution of metabolic flux, resulting in a decrease in the synthesis of 2,3-BDO and an increase in the synthesis of 1,3-PDO and LAC. Fermentation of *K. pneumoniae*  $\Delta$ ALS using glycerol as a substrate resulted in the production of 72.04 g L<sup>-1</sup> of 1,3-PDO, which was a slightly higher than that of the parent strain (Table 3; Fig 4). Inactivation of the 2,3-BDO pathway was speculatively associated with a decrease in the consumption of NADH under aerobic conditions (Fig 1). This increases the available energy (NADH) supply that would otherwise be used by the competing 2,3-BDO pathway under anaerobic conditions. Moreover, LAC was the most abundant byproduct and reached 31.50 g L<sup>-1</sup>, double that of the parent strain. Cell growth was slightly decreased and glycerol utilization was slightly promoted compared to the parent strain. Interestingly, synthesis of *meso*-2,3-BDO was markedly inhibited in the mutant strain but synthesis of 2S,3S-BDO was increased (Fig 5). This indicates that an unknown enzyme catalyzes the conversion of pyruvate to diacetyl. The mutant strain retained ~20% of the ALS activity of the parent strain, and this unexpected residual activity may have contributed to the results. However, the ALS enzyme assay is based on measuring acetoin but not in the presence of acetolactate. The acetoin could be generated by the ALD and this could confound the readout.

Although the yield of 1,3-PDO was increased, the conversion was not promoted to a great degree, mainly due to the accompanying increase in LAC synthesis. Therefore, we predict that blocking LAC synthesis may be the preferred strategy when engineering 1,3-PDO production in *K. pneumoniae*. Our results indicate that 2,3-BDO synthesis competes with the synthesis of 1,3-PDO and LAC. Moreover, since blocking the formation of LAC stimulated 1,3-PDO and 2,3-BDO production in *Klebsiella* species [12, 13], we predict that knocking out ALS in combination with engineering of the LAC pathway would result in a mutant with lower LAC and 2,3-BDO by-products. This was proved by Oh et al. [26] but they did not get even higher 1,3-PDO production with the LAC-ALS dual-mutant. Heterologous expression of pyruvate decarboxylase and aldehyde dehydrogenase efficiently recovered glycerol metabolism in the 2,3-BDO synthesis-defective mutant [27]. They suggested the production of 1,3-PDO could be enhanced by preventing the accumulation of pyruvate.

Three enzymes in the butanediol synthesis pathway are encoded by budB, budA, and budC. *Klebsiella* spp. produces *meso*-2,3-BDO together with a small amount of 2S,3S-BDO. 2S,3S-BDO formation depends on the activity of budC. Butanediol dehydrogenase can catalyze the conversion of diacetyl to S-acetoin and further to 2S,3S-BDO as well as R-acetoin to *meso*-BDO. Diacetyl is a nonenzymatic catalyzed product of  $\alpha$ -acetolactate. BudC is the only known enzyme that catalyzes the conversion of diacetyl to S-acetoin and further to 2S,3S-BDO [28]. The metabolic pathway of the two isomers of 2,3-BDO synthesis in *K. pneumoniae* is shown in Fig 1. In this study, deleting the budB gene not only decreased the activity of ALS, but also decreased *meso*-2,3-BDO and increased 2S,3S-BDO. This phenotype indicates that *meso*-2,3-BDO formation is dependent on the activity of ALS whereas 2S,3S-BDO formation is

inhibited by the activity of ALS. ALS is the first enzyme in the biosynthetic pathways of *meso*-2,3-BDO and 2S,3S-BDO in *Enterobacteriaceae* [17], and catalyzes the conversion of pyruvate to 2,3-BDO. Thus, it was not unexpected to observe a decrease in the formation of *meso*-2,3-BDO in the ALS-deficient mutant strain. The observed increase in 2S,3S-BDO in the mutant strain could be explained by an unknown enzyme that catalyzes the conversion of pyruvate to diacetyl and 2S,3S-BDO. Significantly, the mutation of ALS resulted in the accumulation of a greater amount of 2S,3S-BDO, suggesting this phenomenon could be helpful for 2S,3S-BDO production.

Glycerol metabolism is a typical biological redox pathway. In this study, a 2,3-BDO pathway-deficient mutant of *K. pneumoniae* HLS4 was constructed by knocking out the ALS gene. To investigate the influence of the ALS mutation on glycerol metabolism, ALS activity and various physiological properties including the yield of products and the distribution of metabolites and 2,3-BDO isomers was compared in the  $\Delta$ ALS and wild-type strains. Both the glycerol flux and distribution of metabolites were profoundly altered in the mutant strain in which the biosynthesis of *meso*-2,3-BDO was suppressed but 2S,3S-BDO synthesis was increased, while 1,3-PDO synthesis were slightly increased and LAC synthesis were largely increased. 1,3-PDO concentration and productivity were slightly increased by 4.71% and 4.65%, respectively. The overall conversion of glycerol to 1,3-PDO was not increased significantly due to an accompanying increase in the synthesis of LAC. Further engineering preventing the accumulation of pyruvate may be beneficial to improve 1,3-PDO production and reduce byproducts.

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