Enhanced production of 2,3-butanediol by overexpressing acetolactate synthase and acetoin reductase in *Klebsiella pneumoniae*

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Abstract

Mutants with overexpression of α-acetolactate synthase (ALS), α-acetolactate decarboxylase, and acetoin reductase (AR), either individually or in combination, were constructed to improve 2,3-butanediol (2,3-BD) production in *Klebsiella pneumoniae*. The recombinant strains were characterized in terms of the enzyme activity, 2,3-BD yield, and expression levels. The recombinant *K. pneumoniae* strain (KG-rs) that overexpressed both ALS and AR showed an improved 2,3-BD yield. When cultured in the media with five different carbon sources (glucose, galactose, fructose, sucrose, and lactose), the mutant exhibited higher 2,3-BD productivity and production than the parental strain in all the tested carbon sources except for lactose. The 2,3-BD production of KG-rs in a batch fermentation with glucose as the carbon source was 12% higher than that of the parental strain. Volume 61, Number 6, Pages 707–715, 2014

Keywords: *2,3-BD, acetolactate decarboxylase, acetoin reductase, acetolactate synthase, Klebsiella pneumoniae*

1. Introduction

2,3-Butanediol (2,3-BD), which is also known as 2,3-butylene, is an important chiral compound because of its wide applications [1, 2] in the food, chemical, pharmaceutical, and cosmetic industries [3–5]. 2,3-BD biorefining has attracted significant interest because such a process is more sustainable, environmentally friendly, and economical compared with chemical synthesis [6, 7].

A number of species that can produce 2,3-BD are classified under the genera *Enterobacter*, *Klebsiella*, *Serratia*, and *Bacillus* [1, 8]. The fermentation pathways of 2,3-BD in bacteria have been extensively studied [1, 4, 5]. Numerous studies have investigated the use of metabolic engineering in modifying metabolic pathways to improve 2,3-BD production in *Enterobacter aerogenes* [9], *Bacillus licheniformis* [10], and *Klebsiella oxytoca* [11]. The *ldhA* and *aldA* genes were inactivated in *E. aerogenes* [9] and *K. oxytoca* [11], respectively. These results suggest that 2,3-BD production was improved, the by-product yield was reduced, and the nicotinamide adenine dinucleotide plus hydrogen (NADH) and carbon sources for 2,3-BD biosynthesis were increased. The *ldhB* gene in *B. licheniformis* was disrupted by Wang et al. [10], thereby increasing the efficiency of 2,3-BD production from glucose. However, metabolic engineering focused on 2,3-BD

Abbreviations: *2,3-BD, 2,3-butanediol; ALD, acetaldehyde dehydrogenase; ALDC, α-acetolactate decarboxylase; ALS, α-acetolactate synthase; AR, acetoin reductase; bp, base pairs; DCW, dry cell weight; dNTP, deoxy-ribonucleoside triphosphate; KG1, Klebsiella pneumoniae parent strain; LB, Luria-Bertani; LDH, lactate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide plus hydrogen; PCR, polymerase chain reaction.*

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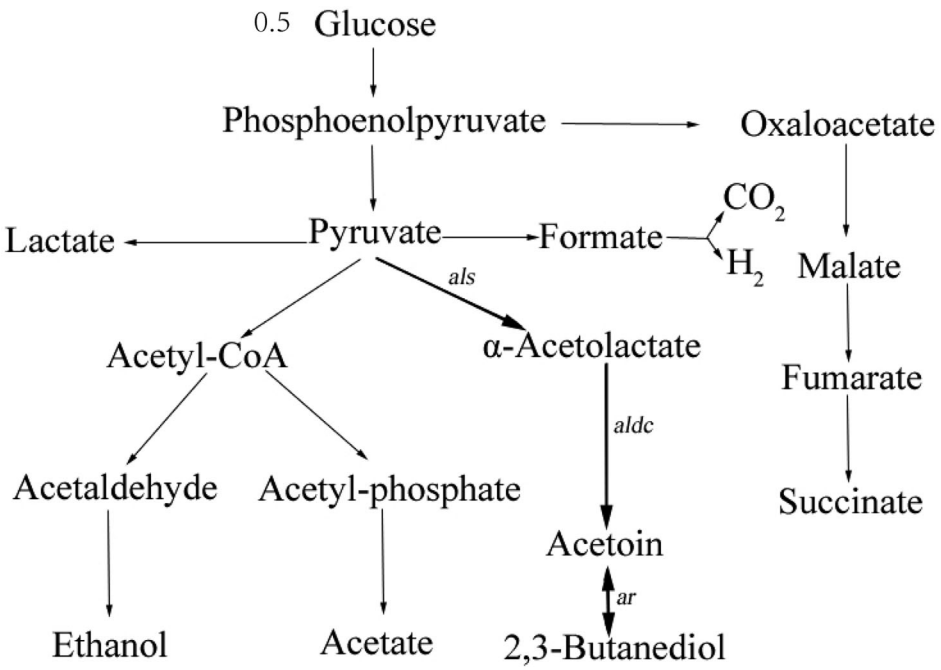

FIG. 1 *Fermentation pathways in Klebsiella pneumoniae (modified from Ji et al. [5]).*

production in *Klebsiella pneumoniae* has not been investigated extensively.

K. pneumoniae is recognized as a useful producer in industrial 2,3-BD production because of the bacteria's wide substrate spectrum, high efficiency, and cultural adaptability [1, 12, 13], and the relevant metabolic pathway is adequately understood. The biosynthesis route of 2,3-BD in *K. pneumoniae* proceeds via pyruvate, acetolactate, and acetoin to 2,3-BD. 2,3-BD production from pyruvate involves three enzymes, namely, α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), and acetoin reductase (AR); these enzymes catalyze the production of acetolactate from pyruvate, acetoin from acetolactate, and 2,3-BD from acetoin [14]. Genetically altering *K. pneumoniae* causes it to be an attractive and useful organism for industrial 2,3-BD production.

In the present study, we attempted to improve 2,3-BD production by enhancing the concentration of key enzymes for 2,3-BD biosynthesis. The pUK18k plasmid was used to overexpress ALS, ALDC, and AR, and the effects of the overexpression on 2,3-BD production in *K. pneumoniae* (Fig. 1) were evaluated. The fermentation results indicated that simultaneous overexpression of ALS and AR resulted in a significant improvement in 2,3-BD yield.

2. Materials and Methods

2.1. Strains, plasmids, media, and cultivation conditions

The plasmids and strains used in this study are listed in Table 1. The *Escherichia coli* strain DH5α was used for the construction and amplification of the plasmids. *K. pneumoniae* KG1 was kindly sent by Zheng et al. [15], which was deposed at China Center of Industries Culture Collection CICC 10781. The *E. coli* strain DH5α was incubated in Luria-Bertani (LB) medium, which was supplemented with kanamycin (30 μg/mL) for plasmid maintenance. The glucose fermentation medium (pH 7.0) was made by combining glucose (80 g/L), yeast extract (10 g/L), KH_2PO_4 (10 g/L), K_2HPO_4 (7.2 g/L), $(NH_4)_2SO_4$ (2 g/L), sodium citrate (4 g/L), and trace element solution (1 mL). *K. pneumoniae* was cultured in the glucose fermentation medium at 35 °C and 150 rpm. The trace element solution contained the following compositions: 3 g/L ethylenediaminetetraacetic acid; 0.09 g/L $CaCl_2{\cdot}2H_2O$; 0.90 g/L $ZnSO_4{\cdot}7H_2O$; 0.60 g/L $FeSO_4{\cdot}7H_2O$; 200 mg/L H_3BO_3; 156 mg/L $MgCl_2{\cdot}2H_2O$; 80 mg/L $Na_2MoO_4{\cdot}2H_2O$; 60 mg/L $CoCl_2{\cdot}2H_2O$; 60 mg/L $CuSO_4{\cdot}5H_2O$; and 20 mg/L KI. The pH of the trace element solution was adjusted to 4.00 by using NaOH and autoclaving.

2.2. Construction of plasmids and recombinant strains

The primers used in this study are listed in Table 2. The total genomic DNA of the *K. pneumoniae* and *E. coli* cells was extracted using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA). ALS (GeneID: 5342277) was amplified by performing PCR using the total genome DNA as template and the primers als-up and als-down, which were designed using the sequence information of ALS of *K. pneumoniae*. The PCR mixture consisted of 1 ng genomic DNA, 0.2 mmol each deoxy-ribonucleoside triphosphate (dNTP), 0.2 μmol each primer, 2 μL rTaq PCR buffer, and 1 unit rTaq DNA polymerase (TaKaRa, Dalian, People's Republic of China)

TABLE 1 *Plasmids and strains used in this study*

Strain, plasmid	*Genotype, properties*	*Source or reference*
Strains		
K. pneumoniae KG1	Parent strain	[15]
KG-ar	KpG containing plasmid pUC-ar	This work
KG-aldc	KpG containing plasmid pUC-aldc	This work
KG-als	KpG containing plasmid pUC-als	This work
KG-dr	KpG containing plasmid pUC-dr	This work
KG-rs	KpG containing plasmid pUC-rs	This work
KG-ds	KpG containing plasmid pUC-ds	This work
KG-drs	KpG containing plasmid pUC-drs	This work
E. coli DH5α	supE44 $\triangle$lacU169 (φ80 lacZ $\triangle$M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	TaKaRa
Plasmids		
pUC18K	*Kam*^{r}, pUC ori, P_{lac}, MCS	[15]
pUC-aldc	Km^r, pUC18K containing *aldc* gene	This work
pUC-ar	Km^r, pUC18K containing *ar* gene	This work
pUC-als	Km^r, pUC18K containing *als* gene	This work
pUC-dr	Km^r, pUC18K containing *aldc* and *ar* genes	This work
pUC-rs	Km^r, pUC18K containing *als* and *ar* genes	This work
pUC-ds	Km^r, pUC18K containing *aldc* and *als* genes	This work
pUC-drs	Km^r, pUC18K containing *aldc*, *als*, and *ar* genes	This work

in a total volume of 20 μL. PCR was conducted at 95 °C for 5 Min, followed by 30 cycles at 95 °C for 40 Sec, 60 °C for 90 Sec, 72 °C for 120 Sec, and 72 °C for 10 Min (final extension step). ALDC (GeneID: 5342278) and AR (GeneID: 5338708) were amplified by performing PCR using the corresponding primers; the PCR procedure was the same as that for ALS except that the annealing temperatures were 62 °C and 58 °C, respectively. All the plasmids were transformed into the parent strain *K. pneumoniae* KG1 in accordance with standard transformation protocol by using electrotransformation [16] and selected by the LB plates containing kanamycin (30 μg/mL), resulting in the recombinant strains.

2.3. SDS-PAGE for bacterial protein analysis

The expression level of the genes in the recombinant and parent strains was confirmed using SDS-PAGE. The cell grown in LB medium containing ampicillin and kanamycin was harvested by performing centrifugation at 12,470*g* for 10 Min, followed by sonication to obtain the crude extracts. The protein concentrations were determined using Bradford assay [17]. Approximately 8 μL of the mixture (containing 6 μL crude extracts and 2 μL 4× protein SDS-PAGE loading buffer) was loaded onto the gel. Electrophoresis was performed with a mini-PROTEAN 3 cell (BIO-RAD, Hercules, CA, USA) apparatus by using 10% SDS buffer at 50 V for 30–40 Min, followed by 120 V for 2–3 H. The gel was stained using Coomassie Blue G250.

2.4. Analytical methods

The biomass was determined using a UV–visible spectroscopy system (8453; Agilent, Palo Alto, CA, USA) at 600 nm with appropriate dilution and was converted to dry cell weight. The intracellular NADH and nicotinamide adenine dinucleotide (NAD^+) concentrations were measured by procedures presented by Ji et al. [18]. The concentrations of glucose, 2,3-BD, acetic acid, lactic acid, alcohol, and acetoin were determined using high-performance liquid chromatography (Agilent 1260 Infinity LC; Agilent, Palo Alto, CA, USA; Shodex RI-101 Refractive Index Detector, G1362A; Agilent, Palo Alto, CA, USA;

TABLE 2 *Primers used in this work*

Primer name	*Primer sequence*
aldc-up	5′-CCGGAATTCCTCTATCAGA CATCGCTC-3′ (*Eco*RI)
aldc –down	5′-CCGGAATTC CCCTTAACTT TCTACGG-3′ (*Eco*RI)
als-up	5′-CCCAAGCTTGGCACCACATCA AACACAAT-3′ (*Hin*dIII)
als-down	5′-CCCAAGCTTTTATTCCCCC ACCATTTCAGT-3′ (*Hin*dIII)
ar-up	5′-CCGGAATTCATGAAAAAAGT CGCACTTGTT-3′ (*Eco*RI)
ar-down	5′-CCCAAGCTTTTAGTTAAACA CCATCCC-3′ (*Hin*dIII)
Test-up	5′-CGGGTTTCGCCACCTCTGA CTTGA-3′

Aminex HPX-87 H Ion Exclusion Column 300 × 7.8 mm^2; Bio-Rad, Hercules, CA, USA) under the following conditions: sample volume, 10 μL; mobile phase, 0.005 M H_2SO_4; flow rate, 0.6 mL/Min; and column temperature, 65 °C.

2.5. Enzyme activity measurements

The ALS activity in the cell extracts was measured as previously described [19]. One unit of ALS activity corresponds to the formation of 1 mmol of acetoin/mg protein/Min. The protein concentration was determined using Bradford assay (1976) with bovine serum albumin as standard [17].

The AR activity was measured as previously described but with a modified reaction mixture containing 50 mM KPi (pH 6.0), 0.2 mM NADH, and 2.5 mM acetoin [20]. One unit of AR activity is defined as the oxidation of 1 μmol of NADH/Min.

The ALDC activity was measured according to the method established by Sone et al. [21]. One unit of ALDC activity is defined as the amount of protein that forms 1 pmol of acetoin/Min.

3. Results

3.1. Enzyme activities of the recombinant *K. pneumoniae* strains

The enzyme activities of the recombinant and parent strains are listed in Table 3. The ALS, ALDC, and AR activities in KG-als, KG-aldc, and KG-ar increased by 1.63, 1.43, and 1.71 times, respectively, over that of the enzyme activity in wild-type *K. pneumoniae* KG1. The corresponding enzyme activities in KG-rs, KG-ds, KG-rd, and KG-rsd also increased relative to that in KG1. The enzyme activities of recombinant *K. pneumoniae* strains indicate that ALS, ALDC, and AR were overexpressed.

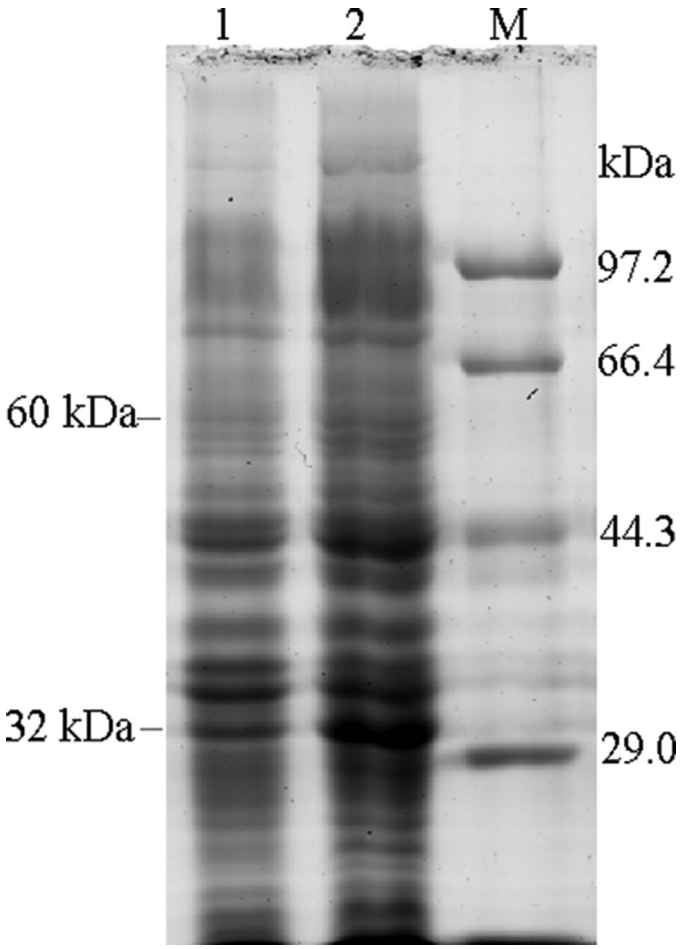

FIG. 2 *SDS-PAGE of proteins in KG-rs and KG1 (pUC18K). M, Marker; 1, KG1 (pUC18K); 2, KG-rs. All strains were cultured in fermentation medium. All cultivations were carried out at 37 °C, 200 rpm, and 200 mL media in a 500 mL shake flask with 5% initial inocula. The recombinant protein expression was carried out at 37 °C for 6 H; when OD_{600} reached 0.5, IPTG was added to 1.0 mmol/L, ALS: 60 kDa, AR: 32 kDa.*

3.2. 2,3-BD production by engineered *K. pneumoniae*

The shake flask fermentation of the recombinant and parent strains was performed at an initial glucose concentration of 67 g/L. As shown in Table 4, 2,3-BD production was almost not increased, and the acetoin yield decreased in the KG-als, KG-aldc, KG-ar, KG-ds, and KG-rds strains. In KG-rs, 2,3-BD production and acetoin yield increased to 24.48 and 2.58 g/L, respectively, after 24 H, and the 2,3-BD yield (g/g Glc) significantly increased from 0.33 to 0.37. Therefore, the KG-rs mutant was selected as the best strain for the fermentation.

3.3. SDS-PAGE of KG-rs mutant

Figure 2 shows the SDS-PAGE results for the protein in the supernatant after extraction. The recombinant AR and ALS were overexpressed by the engineered *K. pneumoniae*. The

TABLE 3 *Comparison of enzyme activity*

	AR activity		ALDC activity		ALS activity	
Strain	*U/mg protein*	*Percentage of wild-type*	*U/mg protein*	*Percentage of wild-type*	*U/mg protein*	*Percentage of wild-type*
KG1	11.73 ± 0.15[a]	1	3.90 ± 0.19	1	0.65 ± 0.05	1
KG-ar	19.08 ± 0.22	1.63	3.98 ± 0.13	1.02	0.71 ± 0.08	1.09
KG-als	11.18 ± 0.14	0.95	3.88 ± 0.13	0.99	1.11 ± 0.08	1.71
KG-aldc	12.59 ± 0.12	1.07	5.56 ± 0.21	1.43	0.63 ± 0.04	0.97
KG-rs	18.93 ± 0.19	1.61	4.12 ± 0.18	1.06	0.89 ± 0.05	1.37
KG-rd	21.46 ± 0.14	1.83	5.98 ± 0.09	1.53	0.56 ± 0.06	0.86
KG-ds	9.59 ± 0.25	0.82	5.78 ± 0.12	1.48	1.22 ± 0.09	1.88
KG-rds	18.62 ± 0.21	1.59	5.63 ± 0.11	1.44	1.07 ± 0.05	1.65

[a]The data are means of three independent experiments. The enzymes were determined when the cell was grown in glucose media for 5 H.

TABLE 4 *Comparison of final concentrations of metabolites, glucose consumption, 2,3-BD productivity, and yield obtained between wild-type and the recombinant strains after 24 H of batch fermentation*

	Consumption of glucose (g/L)	*2,3-BD (g/L)*	*Biomass (g/L)*	*Acetoin (g/L)*	*Lactate (g/L)*	*Acetate (g/L)*	*Ethanol (g/L)*	*2,3-BD yield (g/g Glc)*
KG1	66.70 ± 0.45[a]	21.84 ± 0.11	4.92 ± 0.06	2.47 ± 0.15	4.78 ± 0.35	2.53 ± 0.13	4.18 ± 0.17	0.33
KG-als	58.60 ± 0.56	18.41 ± 0.08	3.11 ± 0.05	0.33 ± 0.04	6.55 ± 0.28	1.33 ± 0.06	3.86 ± 0.09	0.31
KG-ald	66.40 ± 0.90	22.38 ± 0.07	4.10 ± 0.05	1.49 ± 0.18	6.68 ± 0.45	2.19 ± 0.11	4.54 ± 0.14	0.34
KG-ar	63.64 ± 0.70	20.37 ± 0.12	4.26 ± 0.07	0.30 ± 0.05	7.17 ± 0.42	2.67 ± 0.17	3.99 ± 0.11	0.32
KG-rd	62.56 ± 0.62	20.56 ± 0.09	3.44 ± 0.06	0.54 ± 0.02	6.95 ± 0.26	0.97 ± 0.03	4.06 ± 0.11	0.33
KG-rs	66.82 ± 0.43	24.48 ± 0.05	4.82 ± 0.04	2.58 ± 0.19	4.10 ± 0.35	1.66 ± 0.05	5.32 ± 0.19	0.37
KG-ds	62.30 ± 0.67	21.23 ± 0.07	3.60 ± 0.03	0.67 ± 0.05	7.80 ± 0.28	2.24 ± 0.09	3.67 ± 0.12	0.34
KG-rds	63.14 ± 0.78	20.36 ± 0.11	3.52 ± 0.02	0.24 ± 0.02	7.20 ± 0.33	2.21 ± 0.11	4.16 ± 0.12	0.32

[a]The data are means of three independent experiments.

molecular masses of the protein products of AR and ALS were estimated to be 32 and 60 kDa, respectively (NCBI *K. pneumoniae* protein database). The protein bands corresponding to ALS and AR were observed in the samples obtained from the cultures (Fig. 2). Compared with the wild-type strains, ALS and AR were expressed at higher levels in the recombinant strain of KG-rs. The ALS protein band was not obvious, compared with the AR protein band. The reason may be because of the low content of ALS protein in the strain. Results confirmed that the *als* and *ar* genes were overexpressed in *K. pneumoniae* KG-rs.

3.4. Carbon source effects on the *K. pneumoniae* KG-rs mutant

K. pneumoniae effectively utilizes various carbon sources [1, 22]. Five carbon sources, namely, glucose, galactose, fructose, sucrose, and lactose, were selected in analyzing the metabolic effects of the mutant KG-rs (Table 5). As shown in Table 5, 2,3-BD production and glucose consumption were significantly increased and lactate and acetate production was decreased by the KG-rs strain in the flask culture with glucose. The increase in 2,3-BD production and glucose consumption is presumed to be caused by the reduced pH in the mutant

TABLE 5 *Comparison of KG1 and the KG-rs using several carbon sources in a 12H flask cultivation*

Strain:	*KG1*					*KG-rs*				
Carbon source (40 g/L):	*Glucose*	*Lactose*	*Galactose*	*Sucrose*	*Fructose*	*Glucose*	*Lactose*	*Galactose*	*Sucrose*	*Fructose*
Consumed carbon source (g/L)	35.29 ± 0.42[a]	13.18 ± 0.33	36.22 ± 0.27	39.80 ± 0.38	39.60 ± 0.35	35.99 ± 0.45	13.91 ± 0.31	37.80 ± 0.19	39.70 ± 0.22	39.50 ± 0.28
2,3-BD (g/L)	11.90 ± 0.13	4.38 ± 0.05	11.89 ± 0.09	12.96 ± 0.06	13.39 ± 0.18	13.83 ± 0.12	4.08 ± 0.07	12.96 ± 0.11	14.51 ± 0.12	14.67 ± 0.16
Biomass (g/L)	3.88 ± 0.05	2.59 ± 0.09	3.67 ± 0.05	3.52 ± 0.07	4.09 ± 0.04	3.71 ± 0.09	2.74 ± 0.08	3.71 ± 0.06	3.97 ± 0.05	3.87 ± 0.04
Acetoin (g/L)	0.81 ± 0.05	0.71 ± 0.02	0.94 ± 0.04	1.06 ± 0.04	1.17 ± 0.05	0.90 ± 0.02	0.63 ± 0.02	0.43 ± 0.05	1.10 ± 0.07	1.08 ± 0.05
Lactate (g/L)	5.64 ± 0.18	2.78 ± 0.09	4.45 ± 0.12	4.77 ± 0.11	3.91 ± 0.11	5.20 ± 0.17	2.68 ± 0.09	2.57v0.04	4.68 ± 0.08	3.02 ± 0.08
Ethanol (g/L)	3.42 ± 0.11	1.31 ± 0.12	3.35 ± 0.14	4.74 ± 0.16	4.43 ± 0.19	3.89 ± 0.09	1.23 ± 0.10	3.52 ± 0.12	5.27 ± 0.21	4.44 ± 0.11
Acetate (g/L)	0.82 ± 0.04	1.30 ± 0.02	0.43 ± 0.02	0.79 ± 0.03	0.60 ± 0.05	0.75 ± 0.08	1.26 ± 0.07	0.60 ± 0.02	0.99 ± 0.05	0.89 ± 0.07
2,3-BD productivity (g/L H)	0.99	0.36	0.99	1.08	1.12	1.15	0.34	1.08	1.21	1.22
2,3-BD yield (g/g)	0.34	0.33	0.33	0.32	0.34	0.38	0.29	0.34	0.36	0.37

[a]The data are means of three independent experiments.

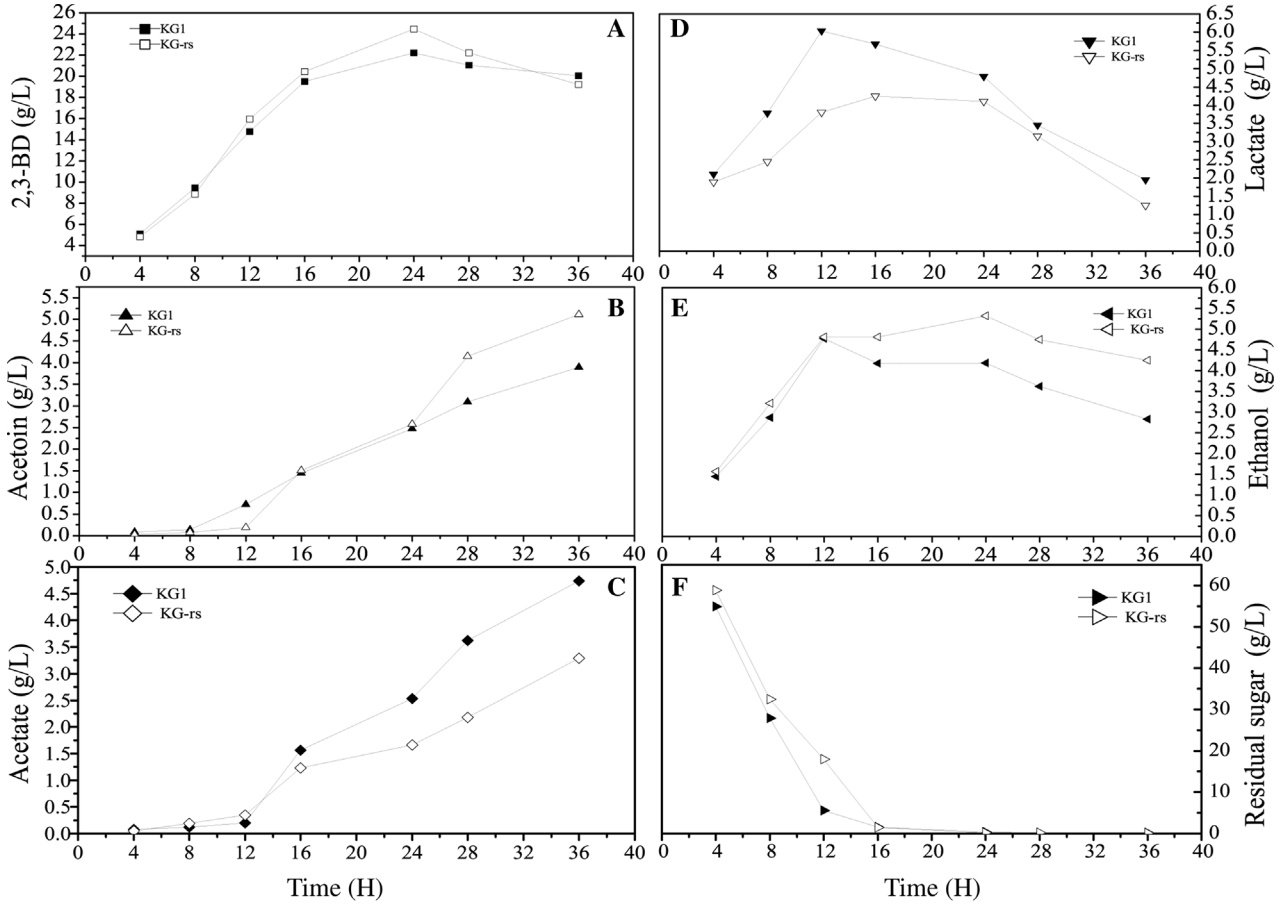

FIG. 3 *Metabolite profiles of the parent strain KG1 and the mutant strain KG-rs in the batch culture. The curves were calculated from the one measurement of three experiments.*

K. oxytoca cultivation media [23]. Therefore, KG-rs underwent less pH shock compared with the wild-type strains; thus, more significant increases in 2,3-BD production were observed in the former.

2,3-BD production by the *K. pneumoniae* KG-rs mutant increased with increasing galactose, fructose, and sucrose (Table 5). Similar to glucose, these carbon sources are consumed in the glycolytic pathway. The biomass production was the highest with fructose in KG1 and KG-rs; however, the cause of this phenomenon remains unclear. The wild-type KG1 was incapable of converting lactose to 2,3-BD because of the strain's low lactose consumption rate. This problem was partly overcome with the mutant KG-rs, which consumed xylose much faster than the wild-type strain. However, the 2,3-BD production from lactose was slightly decreased. Meanwhile, the rate of galactose consumption and 2,3-BD production from galactose with the mutant KG-rs was higher than that with the parent strain KG1. Overall, the mutation KG-rs enhanced 2,3-BD production from all the carbon sources except for lactose.

3.5. Effect of overexpression of ALS and AR on batch fermentations

The growth of the parent strain KG1 and the recombinant strain KG-rs was performed in parallel bath cultures at an initial glucose concentration of 67 g/L. The concentrations of the major metabolites are shown in Fig. 3. As shown in Fig. 3, the recombinant strain produced more 2,3-BD, acetoin, and ethanol, and less acetate and lactate compared with the parent strain, presumably because of the redistributed cellular carbon fluxes. The accumulations of 2,3-BD, acetoin, acetate, lactate, and ethanol in the fermentation broth of the KG-rs differ from those of the parent strain KG1 because of the alteration of the intracellular NADH and carbon fluxes. During the fermentation process, 2,3-BD was produced by the recombinant strain at high rates (Fig. 3A), and the final yields of 2,3-BD, acetoin, and ethanol produced by the recombinant strain were higher than those by the parent strain. However, the final residual sugar was similar for both strains, whereas the acetate and lactate production of the parent strain was less than that of the recombinant strain. These findings suggest that the pathways

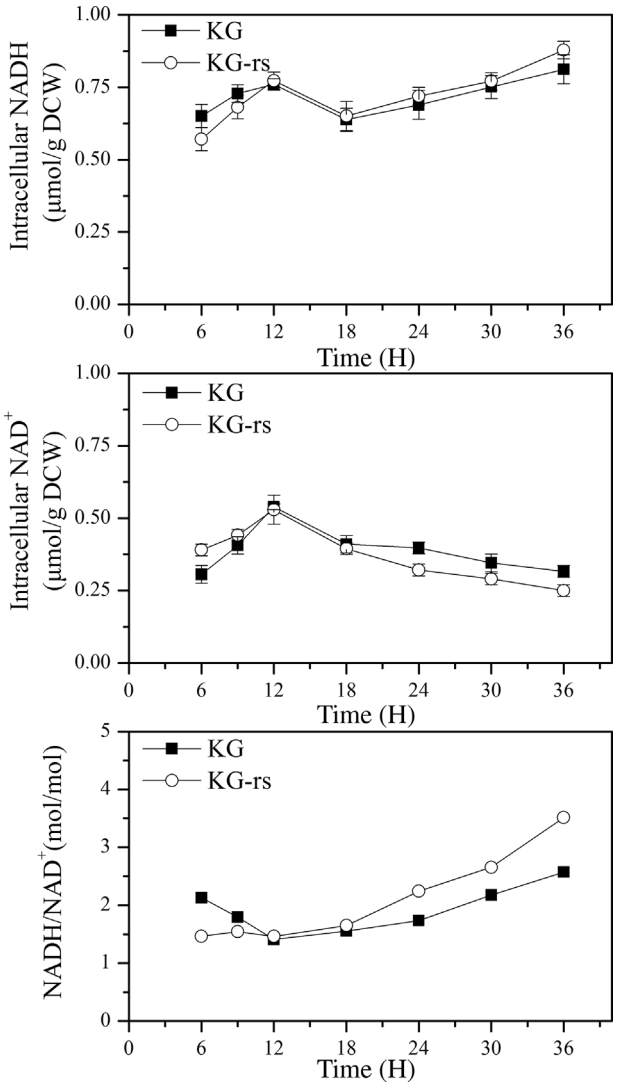

FIG. 4 *Time course profile of nucleotide pools in KG1 and the KG-rs mutant in the batch culture. KG1: the parent strain; KG-rs: the mutants. The curves were calculated from the one measurement of three experiments.*

from pyruvic acid to ethanol, acetate, lactate, and 2,3-BD reacted differently in response to the overexpression of ALS and AR.

3.6. Internal redox state of KG-rs mutant strains

The internal redox state, as reflected by the NADH/NAD^+ ratio, was investigated from 6 to 36 H in the fed-batch fermentation (shown in Fig. 4). As shown in Fig. 4, in KG-rs mutant fermentations, the total dinucleotide pool and NADH/NAD^+ ratio was low in the early exponential period, then increased in the late stationary phase. Compared with *K. pneumoniae* KG1, the NAD^+ level decreased, whereas NADH increased in the fermentation by KG-rs. Thus, the NADH/NAD^+ ratio was much higher in the KG-rs from 12 H. This variation coincides with 2,3-BD flux distribution.

4. Discussion

Metabolic engineering is widely used to improve the properties of microbial strains, such as manipulating the key enzyme levels through the amplification, disruption, or addition of metabolic pathways [18, 24–26]. This study demonstrates that the overexpression of the key enzymes for 2,3-BD biosynthesis caused by a strong promoter increases 2,3-BD production in *K. pneumoniae*. The recombinant strain KG-rs, which overexpressed both ALS and AR, showed higher 2,3-BD production compared with the wild-type strain. The lactate yield was reduced in the KG-rs strain, which may have caused the increase in 2,3-BD production; however, the corresponding enzyme activity (ALS and AR) was only increased by 1.37 and 1.61 times, respectively. This phenomenon may explain the high 2,3-BD yield gained by the reduced lactate production. Lactate is the most abundant by-product that consumes carbon flux and NADH in *K. pneumoniae* and competes with 2,3-BD biosynthesis [9]. Reducing the lactate and acetate yield decreased the acidification rate of the media, which is not favorable to cell growth [9, 27]. Previous studies have reported that lactate and acetate can effectively inhibit *Klebsiella* cell growth, thereby affecting 2,3-BD production [28–30].

KG-als, KG-aldc, and KG-ar, which overexpressed ALS, ALDC, and AR, respectively, did not increase 2,3-BD production but increased the corresponding enzyme activity by 1.63, 1.43, and 1.71 times, respectively, relative to the control strain. Except for KG-rs, all the recombinant mutants exhibited slow growth rate and high by-product yield; such factors may be the reasons why the 2,3-BD yields did not significantly increase. Previous studies have shown that protein overproduction does not always increase the corresponding product yields; on the contrary, this overproduction may be detrimental to cell growth [18, 31]. The same phenomenon was observed in other strains, such as *Saccharomyces cerevisiae* [25], in which only the constitutive expression of protein can dramatically increase the cell growth and improve the fermentation efficiency. The 2,3-BD cycle was another metabolic pathway that produced 2,3-BD [1]. This pathway was characterized by the production of acetyl acetoin and acetyl BD. Therefore, the overexpression of key enzymes at different levels should be regulated by replacing the promoter to improve the product yield. The key enzymes of the 2,3-BD cycle must be investigated in the future.

The effects of key enzyme overexpression were tested using five different carbon sources. The KG-rs mutant exhibited improvement in carbon source consumption rates, biomass production, and 2,3-BD production for all carbon sources except lactose. When lactose was used as the major carbon source for the KG-rs mutant, the 2,3-BD yield and the by-products of lactate, acetate, ethanol, and acetoin was almost reduced; however, the biomass was increased in the KG-rs mutant. In *K. pneumoniae* strains, lactose hydrolysis was the limiting step

because lactose had to first be hydrolyzed by enzymatic processes into monosaccharides, which could then be further converted into BD during the second stage when lactose was used as a major carbon source [32]. Therefore, 2,3-BD production was not improved. The reduced mixed acid production possibly caused the increase in biomass, partly relieving the growth inhibition.

In conclusion, the pursued engineering strategy, which was based on the enhancement of carbon flux toward the desired metabolites, resulted in the development of *K. pneumoniae* strains with high efficiency in 2,3-BD production. The effect of the overexpression of ALS, ALDC, and AR on 2,3-BD production in *K. pneumoniae* was investigated. The 2,3-BD yield of KG-rs increased by 12%, and the productivity from glucose increased from 0.33 to 0.38 g/g, whereas the lactate and acetate concentration significantly decreased. The KG-rs mutant exhibited the highest 2,3-BD yield among the recombinant mutants, and the productivity was from glucose. This study demonstrated that the overexpression of ALS and AR improves 2,3-BD production. The results of this study provide information that is useful for devising industrial biotechnological strategies to achieve high 2,3-BD production in *K. pneumoniae*.

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6. References

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