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Improved 1,3-propanediol production by engineering the 2,3-butanediol and formic acid pathways in integrative recombinant *Klebsiella pneumoniae*

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ABSTRACT

In the biotechnological process, insufficient cofactor NADH and multiple by-products restrain the final titer of 1,3-propanediol (1,3-PD). In this study, 1,3-PD production was improved by engineering the 2,3-butanediol (2,3-BD) and formic acid pathways in integrative recombinant *Klebsiella pneumoniae*. The formation of 2,3-BD is catalysed by acetoin reductase (AR). An inactivation mutation of the AR in *K. pneumoniae* CF was generated by insertion of a formate dehydrogenase gene. Inactivation of AR and expression of formate dehydrogenase reduced 2,3-BD formation and improved 1,3-PD production. Fermentation results revealed that intracellular metabolic flux was redistributed pronouncedly. The yield of 1,3-PD reached 0.74 mol/mol glycerol in flask fermentation, which is higher than the theoretical yield. In 5 L fed-batch fermentation, the final titer and 1,3-PD yield of the *K. pneumoniae* CF strain reached 72.2 g/L and 0.569 mol/mol, respectively, which were 15.9% and 21.7% higher than those of the wild-type strain. The titers of 2,3-BD and formic acid decreased by 52.2% and 73.4%, respectively. By decreasing the concentration of all nonvolatile by-products and by increasing the availability of NADH, this study demonstrates an important strategy in the metabolic engineering of 1,3-PD production by integrative recombinant hosts.

1. Introduction

1,3-Propanediol (1,3-PD) is a useful industrial raw material with numerous applications from polymers to cosmetics, food, lubricants, and medicines. It also serves as a monomer in polycondensation. Nowadays, new types of polyesters, polyethers, and polyurethanes have attracted considerable interest. Compared with other diol-based polymers, such as butanediol, ethylene glycol, or 1,2-propanediol, 1,3-PD-based polymers show better properties and greater stability (Celinska, 2010; Kivisto et al., 2012; Sotenko et al., 2012; Xu et al., 2009a).

The biotechnological production of 1,3-PD shows advantages over the conventional chemical process. The successful heterogenous system for 1,3-PD production is exploited by DuPont and Genencor International. *Escherichia coli* (*E. coli*) K12 is used as a base strain engineered for 1,3-PD production. The recombinant *E. coli* produces 1,3-PD at a titer of 135 g/L in D-glucose fed-batch fermentations (Nakamura and Whited, 2003). Homologous hosts, such as *Klebsiella pneumoniae* (*K. pneumoniae*), are of great significance in industrial application because of their capability to convert biodiesel-derived glycerol into 1,3-PD (Celinska, 2010; Metsoviti et al., 2013; Nemeth and Sevella, 2008).

The microbial production of 1,3-PD consumes NADH, which is produced during the biomass biosynthesis and oxidative pathway of glycerol (Fig. 1) (Celinska, 2010). The formation of by-products will also consume NADH in vivo, compete with the biosynthesis of 1,3-PD from glycerol and finally decrease 1,3-PD yield. 2,3-Butanediol (2,3-BD) is a key competitor to the production of 1,3-PD by *K. pneumoniae* (Celinska, 2010). The biosynthesis of 2,3-BD from pyruvate is considered as a three-step process in *K. pneumoniae* under anaerobic conditions. Pyruvate is firstly catabolised by α-acetolactate synthase to α-acetolactate, which is then decarboxylated by α-acetolactate decarboxylase into acetoin. In the third step, acetoin is converted to 2,3-BD by NAD-dependent acetoin reductase (AR, encoded by the *budC* gene) (Blomqvist et al., 1993; Celinska and Grajek, 2009; Syu, 2001; Wardwell et al., 2001). Moreover, the similarity between 2,3-BD and 1,3-PD will challenge the recovery of 1,3-PD from the mixture of such two diols, thus making purification a bottleneck in the bioprocess (Anand et al., 2011;

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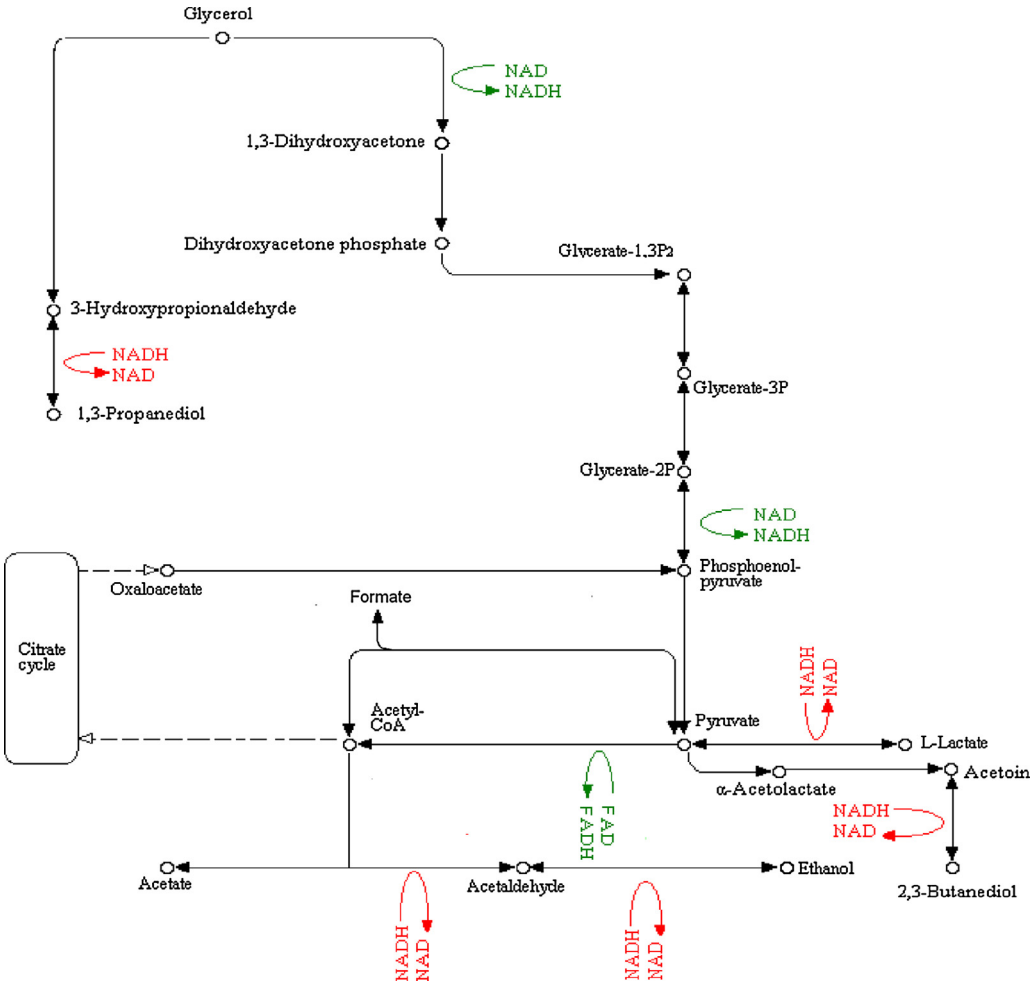

Fig. 1. Pathways of 1,3-propanediol and 2,3-butanediol biosynthesis in *K. pneumoniae*.

Kaur et al., 2012; Zeng and Biebl, 2002). Formate is another toxic by-product in the cultures. It forms when pyruvate is catalysed by pyruvate formate lyase into acetyl-CoA under anaerobic conditions. An advisable way to eliminate formate is to introduce formate dehydrogenase (FDH), which is often utilised in the regeneration of NADH (Shen et al., 2011; Zhang et al., 2009).

Various efforts have been made and applied to produce 1,3-PD efficiently. For example, genes encoding lactate dehydrogenase, α-acetolactate synthase or aldehyde dehydrogenase were knocked out to weaken by-product formation (Kumar et al., 2012; Yang et al., 2007; Zhang et al., 2006, 2012), NADH regeneration system was introduced to increase the availability of redox factors (Zhang et al., 2009) and 1,3-PD synthesis enzymes were overexpressed to strengthen glycerol reductive branch (Zhao et al., 2009a,b). However, none of these efforts increased 1,3-PD production and decreased production of all nonvolatile by-products simultaneously.

In this work, a metabolic engineering strategy was applied to improve the production of 1,3-PD and benefit the separation steps in the downstream in integrative recombinant *K. pneumoniae*. A mutant deficient in AR activity was generated by insertion of tetracycline resistance marker into the *budC* gene. To eliminate formate in culture and further increase the supply of NADH in vivo, the tetracycline resistance marker was replaced by the FDH encoding gene (*fdh*) from *Pichia pastoris*. The engineered integrative recombinant *K. pneumoniae* can produce a higher yield of 1,3-PD and lower yield of all nonvolatile by-products.

2. Materials and methods

2.1. Reagents

Restriction enzymes and ligase were from New England Biolabs. Polymerase and other enzymes were from TAKARA. The kits used for genomic DNA isolation, plasmid isolation, and gene manufacture were purchased from TAKARA. All other chemicals used in this study were of analytical grade or chromatographical grade, and they were bought from Beijing Chemical Company.

2.2. Strains, plasmids and culture media

All strains and plasmids are listed in Table 1. *E. coli* DH5α and the derivatives were cultured in Luria-Bertani medium (LB medium), which contained (grams per liter of deionized water): yeast extract, 5; NaCl, 10; tryptone, 10. *K. pneumoniae* and the derivatives were cultured in kp medium, which contained (grams per liter of deionized water): KH_2PO_4, 1.3; $K_2HPO_4{\cdot}3H_2O$, 4.5; $MgSO_4{\cdot}7H_2O$, 0.2; $(NH_4)_2SO_4$, 3.0; glycerol, 20; yeast extract, 1.0; $CaCl_2$, 0.003; $FeSO_4{\cdot}7H_2O$, 0.01; and 5 mL trace elements. Trace metal solution contained (grams per liter): $CoCl_2{\cdot}6H_2O$, 2.5; $MnCl_2{\cdot}4H_2O$, 15; $Na_2MoO_4{\cdot}2H_2O$, 2.1; Fe (II) citrate 100.8. Ampicillin and/or tetracycline were added to either the LB medium or the kp medium for the selection of the recombinant strains. For cultivating *K. pneumoniae* strains, the pH of the kp medium was adjusted to 7.0 using $CaCO_3$ in flasks or by automatic addition of KOH in the bioreactor.

Table 1
Strains and plasmids.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
K. pneumoniae WT	Wild-type	Lab. collection
E. coli DH5α	Host of plasmids	Lab. collection
K. pneumoniae CT	*K. pneumoniae* Δ*budC*:: *Tet*^{r}	This work
K. pneumoniae CF	*K. pneumoniae* Δ*budC*:: *fdh*	This work
Plasmids		
pMD 18-T	Cloning vector, Amp^r	TAKARA
pBR322	Source of tet, Tet^r	TAKARA
pPK	Source of pk promoter	Lab. collection
pMD18T-budc	pMD18T containing *budc* gene, Amp^r	This work
pMD18T-budc-tet	pMD18T containing *budc-Tet*^{r} gene, Amp^r, Tet^r	This work
pMD18T-budc-fdh	pMD18T containing *budc-fdh* gene, Amp^r	This work

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

Fed-batch cultivations were carried out in 5 L bioreactor (BIOTECH-2002, Shanghai Baoxing Company, China) with a working volume of 2 L. The glycerol concentration in the culture was maintained at around 20 g/L by regulating the feeding rate of the glycerol mixture (700 g/L) manually.

2.3. Construction of plasmids and strains

The strategy for the plasmid and stain constructions was shown in Fig. 2. Total genomic DNA of *K. pneumoniae* was extracted using the kit for genomic DNA isolation (TAKARA). The *budC* gene was amplified by polymerase chain reaction (PCR) using the genomic DNA of *K. pneumoniae* as the template and A1 and A2 as primers:

A1: 5′-CCA TGG ATG CAA AAA GTC GCA CTT GTC-3′ (*Nco*I)
A2: 5′-AAG CTT TCA TAT AGT CGG AAT CCG GGC-3′ (*Hind*III)

The PCR mixture consisted of 8 μL genomic DNA, 10 μL dNTP (2.5 mM), 20 pmol each primer, 2 unit Ex. Taq DNA polymerase and 10 μL Ex. Taq buffer (TAKARA, Japan) in a total volume of 100 μL. PCR reactions were carried out in PCR Express (Techne, England). The PCR program consisted of an initial denaturation (94 °C, 4 min); 30 cycles of denaturation (94 °C, 60 s), annealing (58 °C, 60 s), and elongation (72 °C, 90 s). The 0.7 kb PCR product was cloned into pMD 18-T, yielding plasmid pMD-C.

The tetracycline-resistant gene (*Tet*) was amplified by PCR using plasmid pBR322 (TAKARA, Japan) as the template DNA and pTetF and pTetR as the primers:

pTetF: 5′-TAT GCG GCC GCC ACT CGT GCA CCC AAC TG-3′ (*Not* I)
pTetR: 5′-GTG CTC GAG GCT TCC ATT CAG GTC GAG GT-3′ (*Xho* I)

The PCR mixture and program were similar to the amplification of *budC*. The 1.40-kb PCR product was inserted into the *budC* gene in pMD-C using the *Not* I and *Xho* I sites in *budC*, yielding the plasmid pMD-T.

The DNA fragment containing the disrupted *budC* gene was amplified by PCR using pMD-T as the template DNA and A1 and A2 as the primers. The 1.8 kb PCR product was purified and transformed into the competent cells of *K. pneumoniae* by standard electrical transformation protocol using electrical transformation apparatus (Micropulser^{TM}, Bio-Rad), getting *K. pneumoniae* CT.

The *fdh* was amplified by PCR using *P. pastoris* as the template DNA and pfdhF and pfdhR as the primers:

pfdhF: 5′-CAT ATG AAA ATC GTT CTC GTT TTG TAC TCC-3′
pfdhR: 5′-CTC GAG TGC GAC CTT TTT GTC ATT AC-3′

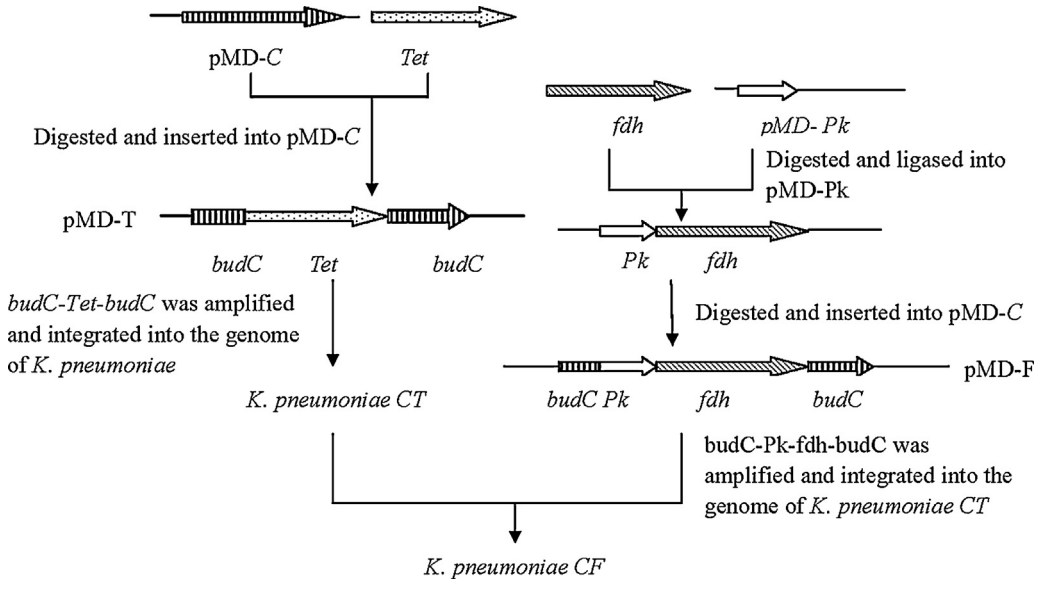

Fig. 2. The strategy for the plasmid and stain constructions.

The 1.1 kb product was digested and inserted into plasmid pMDPK, and then the *fdh* with the Pk promoter was amplified using pFF and pfdhR as the primers:

pFF: 5′-CTA TGC GGC CGC CGT TAT TTT GTC GCC CGC CAT-3′

The 1.3 kb PCR product was inserted into the *budC* gene in pMD-C using the *Not* I and *Xho* I sites, yielding the plasmid pMD-F. Then, the primers A1 and A2 were used to obtain the *budCF*. The product was purified and transformed into the competent cells of *K. pneumoniae* CT by standard electrical transformation protocol using electrical transformation apparatus to obtain *K. pneumoniae* CF. The cells were spread on LB plates containing 25 mM formate. The well-grown cells were selected for further positive colony selection.

2.4. Preparation of cell-free extracts and enzyme activity assay

Briefly, *K. pneumoniae* CF cells were cultured in flasks for 10 h and collected by centrifugation at 12,000 × *g* for 10 min at 4 °C. The cells were washed twice with ice-cold 50 mM Tris–HCl buffer (pH 7.4) and then resuspended in a final volume of 0.5 mL in Tris–HCl buffer (pH 7.4). The cells were lysed by sonication and the crude extracts were centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatants were used for enzyme assays.

AR activity was assayed by measuring the formation rate of NADH in 3 mL reaction mixture contained 10 μL of cell-free extract, 75 mM Tris–HCl (pH 8.0), 2.0 mM NAD, and 0.1 M 2,3-BD. The reactions were recorded as the increase at A340 (UNIC, UV 2000).

FDH activity was also assayed by measuring the formation rate of NADH. However, the 3 mL reaction mixture contained 10 mL cell-free extract, 2.0 mM NAD, 0.1 M sodium formate and 100 mM β-mercaptoethanol in phosphate buffer (10 mM, pH 7.5).

2.5. Measurement of biomass and metabolic products

Biomass was determined by measuring the turbidity of culture at 600 nm. The dry cell weight (CDW) was calculated by the equation below:

$$\mathrm{CDW\,(g/L)} = 0.255 \times \mathrm{OD_{600}} - 0.0176$$

The concentration of glycerol, 1,3-PD, 2,3-BD and other by-products were determined by HPLC (Young Lin, Korea) using the following apparatus and operating conditions: Bio-Rad Aminex HPX-87H column from Bio-Rad with RID and UV detectors; 65 °C oven temperature; and 5 mM sulfuric acid as mobile phase (0.6 mL/min).

3. Results

3.1. Inactivation of AR in K. pneumoniae

The PCR-mediated gene replacement method was used to inactivate AR in *K. pneumoniae*. The *budC* gene from *K. pneumoniae* was cloned into pMD18-T and then disrupted by inserting tetracycline resistance gene *Tet* into the *Not* I and *Xho* I sites of the *budC* gene. After inserting the *Tet* gene, the 150 bp upstream and 263 bp downstream of the disrupted *budC* gene were used as homology extensions. These procedures ensured that the homologous recombinant in *K. pneumoniae* was efficient.

The PCR-amplified 1.8 kb *budCT* was transformed into *K. pneumoniae*. After screening for tetracycline-resistant colonies, several putative *budC* knockout mutants, *K. pneumoniae* CT, were obtained. The PCR products with *K. pneumoniae* CT strains as the template DNA and A1 and A2 as the primers were purified and then subjected to sequencing analysis. Sequencing analysis confirmed that the *Tet* gene was inserted into the *budC* gene of *K. pneumoniae*.

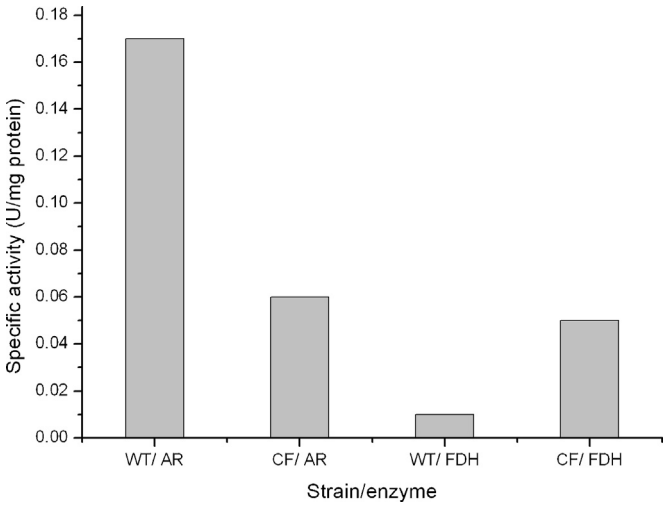

Fig. 3. Enzyme activity determination in the mutant strain *K. pneumoniae* CF and the parent strain *K. pneumoniae* WT.

The *budCF* gene was amplified from pMD-F and then transformed into *K. pneumoniae* CT to replace *Tet* and introduce a FDH. The colonies were sprayed on LB plates with 25 mM sodium formate. Then, colonies with high formate tolerance were screened by colony PCR using primers A1 and pfdhR, confirming that the *Tet* gene was replaced with the *fdh* gene.

The AR and FDH activities of the wild-type (WT) strain and the mutant CF were determined (Fig. 3). The specific activity (0.06 U/mg protein) of AR of *K. pneumoniae* CF was lower than that (0.17 U/mg protein) of the WT strain. This result confirmed that the *budC* gene in CF was disrupted, which inactivated AR. In addition, the specific activity of the FDH of *K. pneumoniae* CF obviously increased from 0.0104 U/mg protein to 0.0530 U/mg protein, which proved the successful insertion of the *fdh* gene and verified the functionality of FDH (Fig. 3).

3.2. AR inactivation triggered the redistribution of metabolic flux in K. pneumoniae

K. pneumoniae CT was fermented in flask without aeration with *K. pneumoniae* as control (Table 2). The mutant strain displayed a relatively low productivity of 2,3-BD. The yield of 1,3-PD from glycerol during the fermentation of *K. pneumoniae* CT also decreased. The titer of 1,3-PD was 6.57 g/L, which was 92.53% of that of the WT strain. This result was different from the results presented in the literature (Zhang et al., 2012). This discrepancy may be attributed to the dramatic decrease in CDW. The production of 2,3-BD decreased from 2.77 g/L of the WT strain to 0.87 g/L of the *K. pneumoniae* CT. In addition, the CDW and ethanol production decreased in *K. pneumoniae* CT, which were 65.20% and 47.56% of those of the WT strain. The final titer of acetic acid in *K. pneumoniae* CT was 16.38% higher than that of the WT strain.

Clearly, the inactivation of AR triggered redistribution of metabolic flux in *K. pneumoniae* (Table 2).

3.3. FDH expression improved 1,3-PD production

The 1,3-PD production by *K. pneumoniae* CF with AR inactivation and FDH expression was higher than that by WT or CT under the same fermentation conditions. When potassium formate was added to the medium, cell growth and 1,3-PD formation by CF were inhibited (Table 2). The fermentaton of CF without potassium formate in flask showed a titer of 8.89 g/L and a yield of 0.74 (Table 2), which is higher than the theoretical yield of 1,3-PD (0.72 mol per

Table 2
The flask fermentation results.

Strain	Concentration of potassium formate added at 0 h (mM)	Concentration of potassium formate added at 12 h (mM)	Titer of 1,3-PD (g/L)	Yield of 1,3-PD to glycerol (mol/mol)	Titer of 2,3-BD (g/L)
WT	0	0	7.10	0.59	2.77
CT	0	0	6.57	0.55	0.87
CF	0	0	8.89	0.74	1.22
	0	25	6.99	0.55	0.42
	25	0	6.20	0.65	0.80
	25	25	6.28	0.63	0.94
	50	50	5.13	0.36	0.98

glycerol). This change probably contributed to the introduction of FDH, which served as an additional NADH generator and the cleavage of formate. In the flask shake fermentation, the yield of 1,3-PD and 2,3-BD of CF were higher than those of CT. The cultures with extrinsic formate produced less 1,3-PD, especially those with high concentrations.

The results of fermentation (Figs. 4 and 5) revealed that the intracellular metabolic flux was redistributed (Table 3). The final titer and yield of 1,3-PD of the CF strain in 5 L reactor reached 72.2 g/L and 0.569 mol/mol, respectively, which were 15.9% and 21.7% higher than those of WT strain, respectively. The titers of 2,3-BD and formic acid decreased by 52.2% and 73.4%, respectively. Decreased concentrations of acetic acid, lactic acid and ethanol were also observed in *K. pneumoniae* CF. The titer of nonvolatile by-products was 27.0 g/L, which was 66.7% of that of WT strain.

4. Discussion

The conversion of glycerol to 1,3-PD is a typical oxidoreduction-coupled biosynthesis process (Celinska, 2010; Skraly et al., 1998; Zeng and Biebl, 2002). As one of the major by-products, 2,3-BD competes for NADH consumption and hampers the purification of 1,3-PD (Nemeth and Sevella, 2008). The formate in the medium inhibits the growth of *K. pneumoniae*, thus limiting the productivity of 1,3-PD.

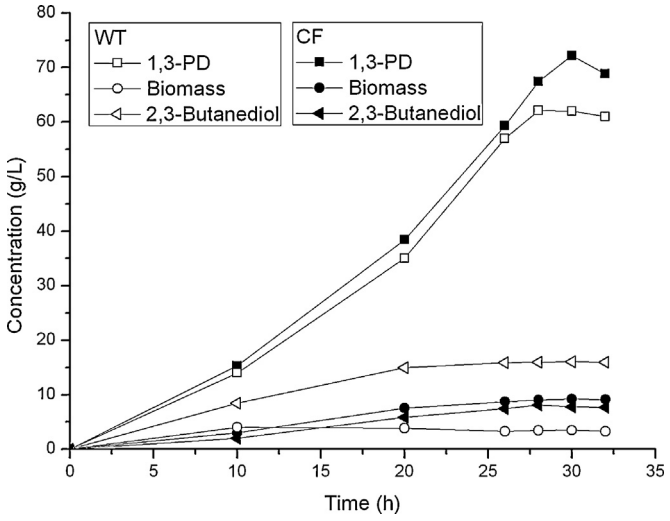

Fig. 4. Metabolites profiles of the mutant strain *K. pneumoniae* CF. Fed-batch cultivations were carried out in a 5-L bioreactor using kp medium. The culture was aerated with nitrogen (1 L/L min) at 20 h to change fermentation from microaerobic conditions to anaerobic conditions. The glycerol concentrations in these cultures were maintained around 20 g/L by using the glycerol feeding strategy determined based on preliminary experiments.

The 2,3-BD-deficient mutant (CT) did not show better performance in producing 1,3-PD compared with the WT stain. This result might be attributed to the flexibility of branch points at pyruvate and acetyl-CoA (Xu et al., 2009b; Yang et al., 2007; Zhang et al., 2006, 2008). Pyruvate formate lyase catalysed the NAD-independent conversion from pyruvate to acetyl-CoA under anaerobic conditions, and then formate formed. When formate formed, no redox factor was generated in the conversion from pyruvate to acetyl-CoA and the cell growth was repressed. Pyruvate was mostly catalysed to acetate, formate and lactate when the 2,3-BD branch was blocked. Thus, the concentrations of acids increased, which influenced the growth of the cells. Similar results were also observed in D-lactate or ethanol deficient mutant (Xu et al., 2009b; Zhang et al., 2006). For example, the final titer of acetic acid in the ethanol deficient strain was 131.2% of that in the wild-type strain (Zhang et al., 2006).

Low biomass resulted in relatively low 1,3-PD productivity. The effects of these by-products on the growth of *K. pneumoniae* were studied. The acid in the medium showed a significant inhibitory effect on cell growth (He et al., 2013). To eliminate the formate and improve the titer and productivity of 1,3-PD by CT, FDH was introduced. The expression of FDH in *K. pneumoniae* decreased the concentration of formate by 73.4% and increased the production of 1,3-PD. Similarly, Zhang et al. introduced the FDH of *Candida boidinii* into *Klebaiella oxytoca* (*K. oxytoca*), and improved the molar yield of 1,3-PD by 17.3% (Zhang et al., 2009).

The inactivation of AR and the expression of FDH could trigger the redistribution of metabolic fluxes. Formic acid is not considered as the main by-product because of its low concentration. However, in the present study, the formate and acetate in the culture dramatically influenced the growth of cells. The increased formic acid decreased cell growth and 1,3-PD production. Therefore, no formic acid was added to the 5 L fermentation culture, and the formate catabolised from the pyruvate became the substrate for formate dehydrogenase. The CDW increased after AR inactivation and FDH expression. This change may be attributed to several reasons. First, the decreased acid resulted in increased CDW. The increased biomass consumed part of the glycerol. Second, two enzymes participated in the anaerobic metabolism of pyruvate: pyruvate dehydrogenase and pyruvate formate lyase (Carere et al., 2008; Nnyepi et al., 2007; Takahata et al., 2008). The introduced FDH cooperated with the latter enzyme to convert pyruvate to CO_2. The exhalant CO_2 evaporated from the culture, and the pyruvate flux to the other by-products decreased. The decreased by-products further improved cell growth.

Most importantly, the yield of 1,3-PD from glycerol increased because of the more efficient consumption of glycerol in the reductive branch. In 5 L bioreactor, the CDW was 164.9% higher than that of the WT strain. This finding may be attributed to the lower concentration of formic acid and 2,3-BD during fermentation process. Although the major pathway for 2,3-BD production was disrupted, 2,3-BD was still detectable in the medium. The reason behind this observation is that 2,3-BD can be formed

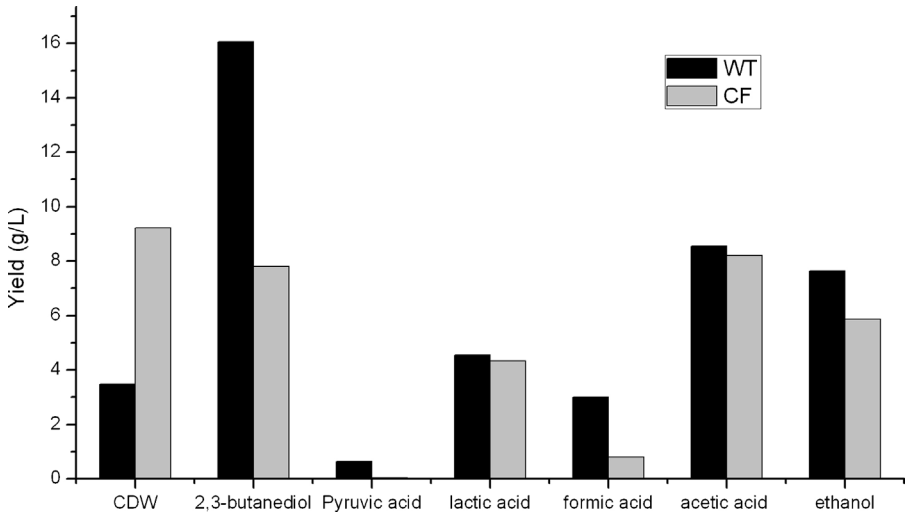

Fig. 5. Comparison of by-product production using different WT and CF.

Table 3
The metabolic fluxes of glycerol in WT and CF (mol/mol glycerol).

Strain	Flux to						
	1,3-PD	2,3-BD	Biomass	Acetate	Lactate	Ethanol	Pyruvate
WT	0.467	0.204	0.0197	0.0815	0.0289	0.0952	0.00416
CF	0.569	0.104	0.0547	0.0819	0.0287	0.0764	0.0002
Fluxes redistribution ratio	21.8%	−49.0%	177.6%	0.5%	−0.69%	−19.7%	−95.2%

Negative data meant a decrease in glycerol flux.

by other short chain dehydrogenases with catalytic activities to convert acetoin. The result of gene blast showed that *budC* had certain similarities with the genes encoding 3-ketoacyl-(acyl-carrier-protein) reductase, 3-oxoacyl-(acyl carrier protein) synthase, NAD-dependent epimerase/dehydratase family protein, putative short-chain alcohol dehydrogenase and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. Some of them probably catalyse the conversion from acetoin to 2,3-BD.

The inactivation of genes participating in the formation of by-products and introduction of NADH regeneration are important strategy in the metabolic engineering of 1,3-PD production. However, when one of the above strategies was employed, the production of by-products is prone to increase (Xu et al., 2009b; Zhang et al., 2006, 2009, 2012). For example, the yield of by-products per CDW in 2,3-BD pathway-deficient mutant increased nearly twofold (Zhang et al., 2012). In another research, after introducing FDH to *K. oxytoca*, molar yields of ethanol and lactate were 42.7% and 18.7% higher than those of parent strain, respectively (Zhang et al., 2009). Thus, the strategy in this work implies the possibility to decrease the production of all nonvolatile by-products. The flux of glycerol shifted to 1,3-PD by sufficient NADH, and the pyruvate was catabolised to alcohol, lactate, acetate, volatile gas, etc. The declined titer of nonvolatile by-products facilitated the purification of 1,3-PD in the downstream process.

A strain with high yield of 1,3-PD and low yield of by-products is possible to be constructed. Pyruvate can be totally channeled to TCA cycle and acetate by blocking the pathways of nonvolatile by-products. FDH was introduced to cleave formate and increase 1,3-PD yield. In an ideal fermentation process, only the target product and the substrate exist in the cultures, thus benefiting downstream purification.

In addition, the integrative recombinant strain is steady and requires no selection markers to maintain plasmid copy number. It maintained homologous or/and heterogenous pathway expression and lack of metabolic burden imposed by plasmids. The integrated way also eliminates the side effects of introducing drug-resistance plasmids in the potentially pathogenic microorganisms. The plasmids can only carry a limited number of genes, whereas multiple heterogenous genes can be integrated into the strain genome. The recombinant strain can be further used for the knockout of homologous enzymes and for the knock in of homologous/heterogenous enzymes. Then, the successful strategies utilised by Dupont, such as deletion of glycerol dehydrogenase and triosephosphate isomerase, as well as introduction of galactose permease and glucokinase (Nakamura and Whited, 2003) can also be applied in *K. pneumoniae*.

5. Conclusions

In this work, AR inactivation and FDH expression in integrative recombinant *K. pneumoniae* were successfully achieved. Inactivation of AR resulted in decreased CDW to 65.20% and increased production of acetate by 16.38%. After introducing FDH, 1,3-PD production by the integrative strain increased by 15.9%, 2,3-BD production decreased by 52.2%, and the titer of nonvolatile by-products decreased to 66.7% of that of WT strain. This engineering approach was proven efficient in improving the yield of 1,3-PD and in reducing the downstream cost.

Acknowledgements

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