Reprogramming the metabolism of *Klebsiella pneumoniae* for efficient 1,3-propanediol production

Weijian Wang¹, Xiao Yu¹, Yongjun Wei², Rodrigo Ledesma-Amaro³, Xiao-Jun Ji^{1, 2*}

¹College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, No. 30 South Puzhu Road, Nanjing 211816, People's Republic of China

²Key Laboratory of Advanced Drug Preparation Technologies, Ministry of Education, School of Pharmaceutical Science, Zhengzhou University, No 100 Kexue Avenue, Zhengzhou 450001, People's Republic of China.

³Department of Bioengineering and Imperial College Centre for Synthetic Biology, Imperial College London, London, SW7 2AZ, U.K.

*Correspondence: xiaojunji@njtech.edu.cn (X.-J. Ji)

Abstract

The production of 1,3-propanediol (1,3-PD) from glycerol by *Klebsiella pneumoniae* is limited by synthesis of numerous byproducts. Among them, the accumulation of acetate has the largest negative impact on the fermentation performance. To address the acetate overflow caused by knocking out lactate dehydrogenase, alcohol dehydrogenase and succinate dehydrogenase, several metabolic engineering manipulations were conducted. First, acetate was reduced through enhancing the acetate assimilation pathway by overexpressing heterologous acetyl-CoA synthetase. Then, the polyhydroxybutyrate (PHB) synthesis pathway was introduced to further reprogram the intracellular carbon metabolism. As a result, the best performed strain Kpr-6 produced up to 91.2 g/L extracellular 1,3-PD and 2.56 g/L intracellular PHB which can be easily separated from each other, while the acetate was dramatically reduced. The metabolic engineering strategies developed in this study would be helpful for constructing the microbial cell factory for other similar bio-based chemical production.

Keywords: 1,3-Propanediol; Metabolic engineering; *Klebsiella pneumoniae*; Acetate overflow; Polyhydroxybutyrate

Highlights:

- *K. pneumoniae* was engineered to improve the production of 1,3- PD.
- Overexpression of acetyl-CoA synthase promoted acetate assimilation.
- PHB synthesis pathway was used to further reprogram the *K. pneumoniae* metabolism.
- The engineered strain co-produced 91.2 g/L extracellular 1,3-PD and 2.56 g/L intracellular PHB that could be easily separated.

1. Introduction

As a versatile bio-feedstock, glycerol is widely used in the production of chemicals, polymers and fuels (Pagliaro et al., 2007). With the rise of the biodiesel industry, large amounts of glycerol are being generated each year and pose an environmental burden (Kumar et al., 2019; Lama et al., 2017). Among the value-added products that can be produced from glycerol, 1,3-propanediol (1,3-PD) is one of the most attractive chemicals, and its desirable properties have long been known (Kumar et al., 2018; Sun et al., 2016). Since the applications of this compound continue to expand, the market demand for 1,3-PD has increased significantly. In particular, 1,3-PD can be polycondensed as a monomer with terephthalic acid to form poly(trimethylene terephthalate) (PTT), which has great potential in carpets, textile fibers, clothing, automobile lining, and other industrial applications (Guo et al., 2017; Ji et al., 2009; Kaur et al., 2013).

Many microorganisms, such as *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium pasteurianum*, *Clostridium butyricum*, *Enterobacter aerogenes*, and *Lactobacillus reuteri* can naturally metabolize glycerol to produce 1,3-PD (Biebl et al., 1999; Da Silva et al., 2009; Zhang et al., 2019b). Among them, *K. pneumoniae* has attracted great attention due to its glycerol biotransformation ability and unexpectedly high glycerol tolerance (Vivek et al., 2017). In *K. pneumoniae*, the synthesis of 1,3-PD relies on the reductive pathway, which is one of the branches in the glycerol dismutation process (Yun et al., 2018). The entire biocatalytic conversion process encompasses the conversion of glycerol to 3-hydroxypropionic aldehyde (3-HPA) by coenzyme B₁₂-dependent glycerol dehydratase (EC 4.2.1.30), and NADH-dependent reduction under the catalysis of 1,3-PD oxidoreductase (EC 1.1.1.202) (Xu et al., 2009). By contrast, the glycerol oxidation pathway causes the formation of several by-products, including 2,3-butanediol, acetate, lactate, ethanol, and succinate (Xin et al., 2017). This process is functionally important for balancing intracellular carbon and cofactor metabolism while providing energy and small molecules for cell growth.

However, excessive accumulation of byproducts is detrimental for industrial production because it reduces the substrate utilization efficiency, eventually leading to a decline in 1,3-PD yield, as well as increasing the difficulty of product separation and purification (Cheng et al., 2005; Zhang et al., 2019a).

Metabolic engineering has been used to increase the titer, yield and productivity of 1,3-PD by eliminating competing pathways and enhancing the expression of rate-limiting enzymes (Celinska, 2010). For example, an engineered *K. pneumoniae* lacking lactate dehydrogenase (LDH, EC 1.1.1.28) and alcohol dehydrogenase (ADH, EC 1.1.1.1) produced significantly less lactate and ethanol, while producing more 1,3-PD and acetate (Ko et al., 2017). However, the resulting high concentration of acetate in the culture medium is toxic to microorganisms because the protonated acetic acid can cross the cell membrane and destroy the transmembrane pH gradient (Luli and Strohl, 1990). Most importantly, the separation of acetate in the post-treatment process usually requires complex procedures such as ion exchange or electrodialysis, which will undoubtedly increase production costs.

Attempts to eliminate acetate accumulation by blocking the acetate synthesis pathway were unsuccessful, because the lack of this pathway will seriously damage the growth of bacteria (Lin et al., 2015). According to previous reports, acetate can be converted to acetyl-CoA through acetate assimilation pathway by acetyl-CoA synthetase (ACS, EC 6.2.1.1), encoded by *acs* gene (Bernal et al., 2016; Huang et al., 2018). That is to say the uptake and secretion of acetate in *K. pneumoniae* is a dynamically regulated process that is closely linked to the cell growth and intracellular metabolism balance. Based on this, we speculated that a reduction of the acetyl-CoA pool will drive the metabolic flux from acetate to acetyl-CoA through the acetate assimilation pathway (Krivoruchko et al., 2015). The polyhydroxybutyrate (PHB) synthesis pathway from *Ralstonia eutropha* is the most thoroughly studied, and it involves three enzymes that catalyze the conversion of acetyl-CoA to PHB while consuming NADPH (Kumar et al., 2018). This stored PHB provides the cells

with a source of carbon and energy under nutrient limitation and enhances their tolerance to environmental stresses (Chen et al., 2019). At the same time, as eco-friendly bioplastics, PHB has great economic value and many potential applications, such as packaging materials, 3D printing materials (Li et al., 2017). When this pathway was introduced into *E. coli*, the acetate concentration in the culture medium was reduced by approximately 66% (Carlson et al., 2005), which is basically in line with our expectations.

In this study, we aimed to regulate the byproduct synthesis of *K. pneumoniae* to improve the yield and efficiency of 1,3-PD production from glycerol. Among the byproducts, the excessive accumulation of acetate will inevitably have an adverse effect on bacterial fermentation and product separation. In view of this, the acetate assimilation pathway favoring acetyl-CoA formation from acetate was strengthened to regulate by-product metabolism. Then, the PHB synthesis pathway, with acetyl-CoA as an intermediate, was reconstructed to change the direction of carbon fluxes in a way that further reduces acetate formation. The relevant metabolic pathways of the resulting engineered *K. pneumoniae* are shown in Fig. 1.

2. Materials and methods

2.1 Strains, media, and culture conditions

All the strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* ATCC 25955 which is insensitive to oxygen was used to produce 1,3-PD from glycerol (Huang et al., 2002). *Escherichia coli* DH5 α was used as a cloning host, and *E. coli* S17-1 was used for plasmid transfer via conjugation. During the construction of recombinant strains, all bacteria were grown at 37 °C in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl). Chloramphenicol (25 μ g/mL) and ampicillin (100 μ g/mL) were added to the medium when necessary. The shake flask medium used for *K. pneumoniae* cultivation was modified from a previously described medium (Zhang et al., 1993), and contained 20 g/L glycerol, 7

g/L yeast extract, 7 g/L $K_2HPO_4 \cdot 3H_2O$, 2 g/L KH_2PO_4 , 1.25 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, and 1 ml/L trace elements solution. The trace elements solution contained 3.2 mg/L $CaCl_2 \cdot 2H_2O$, 3.8 mg/L $ZnCl_2$, 30.0 mg/L $FeCl_3 \cdot 6H_2O$, 11.14 mg/L $MnCl_2 \cdot 4H_2O$, 0.96 mg/L $CuCl_2 \cdot 2H_2O$, 2.64 mg/L $CoCl_2 \cdot 6H_2O$, 0.35 mg/L H_3BO_3 , and 24.5 μ g/L $Na_2MoPO_4 \cdot 2H_2O$. For shake flask fermentation, strains were cultured in 250 mL flasks containing 50 mL medium at 37°C and 160 rpm. The initial OD_{600} was 0.05. The initial pH of the medium was adjusted to 7.0 using 2 M NaOH. Chloramphenicol (20 μ g/mL) was added to the medium to maintain plasmid stability as required. If necessary, isopropyl- β -D-thiogalactoside (IPTG) with final concentration of 1 mM was added into culture medium when the value of OD_{600} reached 0.6-0.8.

2.2 Construction of plasmids and mutant strains

The pKR6K suicide vector was used to construct *K. pneumoniae* mutants by homologous recombination. *E. coli* S17-1 was used to propagate pKR6K and transfer the plasmid into *K. pneumoniae* by conjugation to achieve gene knockout or integration as described previously (Wang et al., 2014). For gene knockout, the up- and downstream homologous sequences of *adhE* (GenBank accession no. CDO15060) were cloned from the genome of *K. pneumoniae* ATCC 25955 using the primer pairs *adhE*-F/*adhE*-F1 and *adhE*-R/*adhE*-R1, respectively. Then, the two fragments were ligated into pKR6K digested with *Pst*I and *Eco*RI by one-step cloning, producing pKR6K- Δ *adhE*. *E. coli* S17-1 harboring the pKR6K- Δ *adhE* plasmid was used as the donor in conjugation with *K. pneumoniae* ATCC 25955 to construct the *adhE* mutant strain. The plasmids for knocking out *ldhA* (GenBank accession no. CDO14265), *frdA* (GenBank accession no. CDO16603) and *aceA* (GenBank accession no. CDO16762) were constructed analogously. The plasmid pKR6K-*frdA*:: P_{pc} -acs_{ap} was used for insertion of the target gene P_{pc} -acs_{ap} into the *frdA* site. The constitutive *pc* promoter and codon-optimized sequence encoding ACS from *Acetobacter pasteurianus* was synthesized by Genewiz (Suzhou, China) (Table S2). The *pc* promoter was previously

shown to be more effective than the tac promoter (Xu et al., 2013). The left and right homologous fragments of the *frdA* gene were PCR-amplified using the primer pairs *frdA*-up1Q-F/*frdA*-up1Q-R and *frdA*-down1Q-F/*frdA*-down1Q-R, respectively. The $P_{pc-acSap}$ fragment, which was amplified using $Pc-ACSapJ$ -F/ $Pc-ACSapJ$ -R, was integrated with the homologous fragments between the *Pst*I and *Eco*RI sites of pKR6K. All cloning primers used in this study are listed in Table S1.

The pDK7 vector was used for gene expression in *K. pneumoniae*. The plasmid pQKZ103 containing the *Ralstonia eutropha* PHB synthesis operon was kindly donated by professor Qingsheng Qi (Kang et al., 2008). To construct pDK7-phbCBA, the pDK7 and pQKZ103 plasmids were digested with *Xba*I and *Hind*III, after which the *phbCBA* operon was integrated into the linearized pDK7 plasmid using T4 ligase. The recombinant plasmid was introduced into the host by electroporation (Jeong et al., 1998). The $P_{pc-acSap}$ cassette was amplified by PCR using the primers $pc-acSap$ -F/ $pc-acSap$ -R and ligated into pDK7 digested with *Xba*I and *Hind*III to generate pDK7- $P_{pc-acSap}$. To construct pDK7- $P_{pc-acS_{kp}}$, the *pc* promoter and *acS_{kp}* gene (GenBank accession no. CDO16679) were respectively amplified using the primer pairs *pc*-F/*pc*-R and *acskp*-F/*acskp*-R, respectively, and ligated into pDK7 digested with *Xba*I and *Hind*III.

2.3 Fed-batch fermentation

Fed-batch fermentation was performed in a 5 L stirred bioreactor (T&J-Btype, J Bio-engineering Co., Ltd, Shanghai, China) with a working volume of 3 L at 37 °C, 200 rpm and 1.0 vvm (volume of air per reactor volume per minute). The pH was kept at 7.0 via the automatic addition of 4 M NaOH. The fed-batch fermentation medium contained 20.0 g/L glycerol, 1.0 g/L yeast extract, 6.0 g/L $K_2HPO_4 \cdot 3H_2O$, 2.0 g/L KH_2PO_4 , 5.0 g/L $(NH_4)_2SO_4$, 0.32 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L $C_6H_5Na_3O_7 \cdot 2H_2O$ and 1.0 ml/L trace elements solution. The components of trace elements solution are the same as described above. The seed culture was grown in shake flask medium at 37°C and 200 rpm on a rotary shaker for 12 h, and then the seed culture (5%, v/v) was used

to inoculate into the bioreactor. When the initial glycerol concentration dropped below 2 g/L, a 60% glycerol feed solution was supplemented to maintain the glycerol concentration in the range of 20-30 g/L. The glycerol supplementation rate was controlled to increase the glycerol concentration by 3-5 g/L every 2 h.

2.4 Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). The content of PHB was determined by gas chromatography (Agilent 8860, Agilent Technologies, USA) after methanolysis of lyophilized cells in chloroform as described before (Wang et al., 2012). Cells were harvested by centrifugation at 10000 × g for 10 min, and the supernatant was used for analysis. The concentrations of 1,3-PD, 2,3-butanediol, acetate, succinate, ethanol, lactate and glycerol were determined by high-performance liquid chromatography (HPLC) (Summit P680, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; AminexHPX-87 H Ion Exclusion Column 300 mm × 7.8 mm, Bio-Rad, USA). The injection volume was 10 µL, the mobile phase consisted of 5 mM H₂SO₄ at a flow rate 0.6 mL/min, and the column temperature was 65 °C.

3. Results and discussion

3.1 Rewiring glycerol metabolism for efficient 1,3-PD production

Under microaerobic conditions, *K. pneumoniae* can convert glycerol into the target product 1,3-PD, with the concomitants generation of byproducts including 2,3-butanediol, ethanol, lactate, succinate, and acetate. The accumulation of these byproducts will cause carbon loss and affect the substrate conversion efficiency. To increase the titer and yield of 1,3-PD, the *ldhA* gene encoding lactate dehydrogenase and *adhE* gene encoding alcohol dehydrogenase were first inactivated in the wild-type strain ATCC 25955, designated as Kpr-1. The shake-flask fermentation results showed that the content of ethanol and lactate in the culture medium was significantly reduced, which was consistent with earlier reports (Ko et al., 2017). Among all

byproducts, 2,3-butanediol is the most abundant and hinders the purification of 1,3-PD due to its similar physical properties (Kumar et al., 2016; Xiu et al., 2008). However, despite the effective reduction of 2,3-butanediol accumulation, the further inactivation of α -acetolactate decarboxylase (EC 4.1.1.5) or α -acetolactate synthase (EC 2.2.1.6) that governed the 2,3-butanediol biosynthesis resulted in a decrease of biomass and 1,3-PD production (data not shown). The 2,3-butanediol synthesis pathway is of great significance for the regeneration of cofactor NADH and the maintenance of intracellular pH stability. It was reported that the lack of 2,3-butanediol synthesis pathway will lead to excessive accumulation of acid (especially pyruvate) and a decrease in pH, which could be the main reason for the reduction of cell growth and 1,3-PD production (Kumar et al., 2016). Similar negative results were obtained when disrupting the acetate synthesis pathway by deactivating the *ackA-pta* gene (data not shown). This might be due to the fact that both metabolic pathways play an important role in cell growth by balancing the intracellular acetyl-CoA pool, energy supply, and synthesis of branched-chain amino acids (Lu et al., 2016; Ohtake et al., 2017). Therefore, the 2,3-butanediol and acetate synthesis pathways were remained complete and the strain Kpr-2 was constructed by further deleting the fumarate reductase gene (*frdA*, GenBank accession no. CDO16603) in strain Kpr-1, which catalyzes the conversion of fumarate to succinate. Compared with the wild type, the strain Kpr-2 produced less lactate, ethanol and succinate, while the production of 1,3-PD increased by 31.1%, reaching 13.9 g/L (Fig. 2B and D). However, as a result of the metabolic perturbation caused by blocking the byproduct synthesis pathways, a large amount of acetate was secreted into the culture medium. The acetate titer of Kpr-2 reached 1.3 g/L at 30 h, whereas no acetate was detected in the wild type (Fig. 2C). Among all the byproducts, acetate has the important influence on bacterial fermentation due to its toxicity. Consistently, the cell growth was affected by intracellular metabolic disorders and the inhibitory effect of acetate. The OD₆₀₀ of Kpr-2 was about 88% of the wild-type value at the end of fermentation (Fig. 2A).

3.2 Overexpression of acetyl-CoA synthase reduced the acetate overflow

The acetate assimilation pathway (catalyzed by acetyl-CoA synthase, encoded by *acs* gene) can effectively catalyze the conversion of acetate into acetyl-CoA in an irreversible reaction (Starai et al., 2004; Yang et al., 2020). Many previous studies have shown that strengthening acetate assimilation pathway can promote the utilization of acetate without significantly affecting cell growth. Therefore, this strategy was widely used to achieve the effective biotransformation of acetate into high-value-added products by microbial cell factories (Noh et al., 2018; Xu et al., 2017). Here, the effects of overexpressing acetyl-CoA synthase from different sources on the metabolism of *K. pneumoniae* were explored. The acetyl-CoA synthase from *Acetobacter pastoris* was first selected due to its excellent ability to utilize acetate (Yang et al., 2016). It was introduced into the engineered strain Kpr-2, resulting in Kpr-2-P_{pc}-acs_{ap}. The nucleotide sequence of *acs*_{ap} was codon-optimized and cloned into the high-copy-number plasmid pDK7 under the control of the constitutive pc promoter. Similarly, the endogenous *acs* gene of *K. pneumoniae* was cloned and the recombinant strain Kpr-2-P_{pc}-acs_{kp} was constructed analogously. The sequence alignment shows that the amino acid sequence similarity of ACS from *K. pneumoniae* and *E. coli* is 92.02%, indicating that the two enzymes have almost the same function (Fig. S1).

As expected, both the recombinant strains harboring the ACS_{ap} and ACS_{kp} showed reduced acetate accumulation in shake-flask fermentations (Fig. 3B). Due to the successful overexpression of *acs* gene, acetate was gradually utilized and finally depleted at 27 h of fermentation. In addition, it can be concluded by analyzing the trend of acetate consumption that the ACS_{ap} was more efficient in utilizing acetate, in agreement with previous reports (Yang et al., 2016). With the consumption of acetate, the cell growth (OD₆₀₀) of the Kpr-2-P_{pc}-acs_{ap} and Kpr-2-P_{pc}-acs_{kp} strains increased by approximately 13.7% and 17.7%, respectively (Fig. 3A). However, the yield of 1,3-PD was reduced by about 11% in both strains, demonstrating that the secretion of

acetate is potentially related to the 1,3-PD production. Besides, the contents of lactate and 2,3-butanediol was also decreased. It is worth noting that the generation of byproducts derived from acetyl-CoA was affected by the overexpression of the *acs* gene, so that the accumulation of succinate and ethanol was increased during the fermentation (Table 2). As a result, the *acs_{ap}* gene was selected to regulate the acetate overflow of *K. pneumoniae* in the subsequent experiment.

3.3 Integrated expression of acetyl-CoA synthase further improved 1,3-PD production

To expand the economic efficiency of industrial production, higher product concentration, conversion rate and volumetric productivity are required (Nakashima et al., 2014). In addition, the purification of target product from by-products accounts for a considerable cost. Previous studies have shown that a large amount of acetate was generated during the fermentation of Kpr-2. The time course of Kpr-2 under fed-batch fermentation was shown in Fig. 4A-F, and the major metabolites included 1,3-PD (83.8 g/L), 2,3-butanediol (33.5 g/L), acetate (10.8 g/L), ethanol (0.41 g/L), lactate (0.40 g/L), succinate (5.58 g/L). Compared with the wild type strain, the final titer of acetate in Kpr-2 increased by 1.5 fold (from 4.2 g/L to 10.8 g/L). To reduce the acetate overflow and improve the strain stability, the P_{pc} -*acs_{ap}* expression cassette was integrated into the genome of Kpr-2 at the *frdA* site, generating Kpr-3, and fed-batch fermentation results was shown in Fig. 4A-F.

After 30 h fermentation, the content of 1,3-PD of strain Kpr-3 increased 13.5% compared with strain Kpr-2 and reached 94.9 g/L (Fig. 4A). Notably, the titer of acetate decreased by 17.6% (from 10.8 g/L to 8.9 g/L) at the end of fermentation (Fig. 4B), indicating that this strategy can effectively regulate the acetate accumulation in *K. pneumoniae*. Lactate production was negligibly affected by the overexpression of acetyl-CoA synthase, whereas 2,3-butanediol production reduced by 13.4% and reached 29.0 g/L (Fig. 4E and F). The final content of succinate (5.0 g/L) and ethanol (0.49 g/L) was detected which is similar to that of the Kpr-2 (Fig. 4C and D).

However, the production of succinate in Kpr-3 was higher than that of the Kpr-2. Further analysis of the trend of acetate accumulation revealed that Kpr-3 produced more acetate in the middle fermentation stage (10-18 h). It can be inferred that the continuous expression of the *acs* gene will inevitably increase the content of acetyl-CoA, thereby activating the acetate synthesis pathway. The massive production of acetate in this stage can provide more energy for cell growth to compensate for the ATP consumed by the acetyl-CoA synthase. In fact, the expression of endogenous *acs* gene is up-regulated by increased flux through acetate synthesis pathway and carbon limitation (Bernal et al., 2016). When the carbon source was continuously consumed, the *acs* gene was expressed and acetate was gradually reused. As shown in Fig. 4B, the content of acetate began to drop from 10 h in Kpr-2. However, with the supplement of glycerol, the acetate production increased under substrate-excess condition. Compared to Kpr-2, the overexpression of *acs_{ap}* in strain Kpr-3 can enhance the absorption of acetate and ultimately reduce the acetate content. According to theoretical calculations, as previously reported, acetate pathway is more favorable for 1,3-PD production (Zeng et al., 1993). If acetate is the only byproduct of glycerol metabolism, the maximum NADH supply for 1,3-PD production can be obtained (Celinska, 2010). Therefore, the production of 1,3-PD improved in this stage (10-18h) and reached plateaus after 20h of fermentation. In conclusion, the final content of 1,3-PD was increased with the integrated expression of acetyl-CoA synthase and the productivity was reached 3.16 g/(L·h). By the way, the similar result was reported in *Clostridium butyricum* that high content of 1,3-PD were associated with high content of acetate and butyric acid (Zhang et al., 2019b). Nevertheless, the acetate production in Kpr-3 was still quite high and need to be further regulated.

3.4 Introducing PHB synthesis pathway further reduced the acetate accumulation and coproduced 1,3-PD and PHB

PHB can be directly synthesized from acetyl-CoA by numerous prokaryotes (Wang et al., 2012). Therefore, the introduction of the heterologous PHB synthesis

pathway is expected to regulate the accumulation of acetyl-CoA derivatives (such as acetate) through intracellular metabolic redistribution. The results of shake flask fermentation showed that the acetate production was reduced after the introduction of PHB synthesis pathway but more succinate was detected in the culture medium (Fig. S2). As an intermediate in the TCA cycle, succinate can also be produced via the glyoxylate bypass, in which isocitrate lyase (EC 4.1.3.1) converts isocitrate into succinate and glyoxylate (Pan et al., 2018). To down-regulate the content of succinate, the *aceA* gene (encoding isocitrate lyase) was further deleted in strain Kpr-3, and the PHB synthesis pathway was introduced, designed as Kpr-6. Fed-batch fermentation was carried out to detect the products of glycerol metabolism. As shown in Fig. 4A, the content of 1,3-PD at 30 h reached 91.2 g/L, which was slightly decreased compared with Kpr-3, but still 18.7% and 8.8% higher than that of the wild-type and Kpr-2 strains, respectively. The final conversion yield of 1,3-PD reached 0.59 mol/mol glycerol and the productivity of 1,3-PD reached 3.06 g/(L·h). As expected, acetate production was dramatically reduced. The final titer of acetate reached 4.3 g/L, which was more than 2-fold decrease than that of the Kpr-3 strain (Fig. 4B). It can be inferred that the introduction of PHB synthesis pathway reduced the metabolic flux of acetyl-CoA into the acetate synthesis pathway. Simultaneously, the intracellular storage PHB was very likely to enhance the strain's tolerance to adverse external environments, such as the accumulation of toxic intermediate metabolite 3-HPA or high concentrations of 1,3-PD and by-products. Therefore, the strain Kpr-6 exhibits strong 1,3-PD production capacity with low acetate content, which is very conducive to industrial production. Lactate production (0.1 g/L) and 2,3-butanediol (29.2 g/L) production of strain Kpr-6 were detected and did not show significant changes compared with Kpr-3 (Fig. 4E and F). Moreover, the accumulation of succinate (from 5.0 g/L to 7.3 g/L) and ethanol (from 0.5 g/L to 1.4 g/L) increased which may be attributed to the disturbance of acetyl-CoA flow (Fig. 4C and D). Succinate has multiple replenishment pathways in the cell, which makes it difficult to effectively reduce its content through single gene editing (Zhou et al., 2020). Correspondingly,

the content of intracellular PHB reached 2.56 g/L which is 34.2% of the dry cell weight at the end of fermentation (Fig. 5). Since PHB was present in the cells in the form of intracellular polymer granules, the conversion of acetate into this product not only reduces the difficulty of product separation, but also increased the economic value of the overall bioprocess.

In general, the application of the metabolic engineering strategies described in this study effectively reduced the accumulation of acetate. Most importantly, the results confirm the feasibility of co-producing 1,3-PD and PHB, which can be easily separated from each other. Although the content of succinate and ethanol had slightly increased, the economic benefits brought by the co-production of 1,3-PD and PHB were obviously higher.

4. Conclusions

In this study, metabolic engineering strategies were used to reprogram the metabolism of *K. pneumoniae* to reduce the acetate accumulation for efficient 1,3-PD production. By integrating the heterologous acetate assimilation pathway, the production of acetate was reduced, and the titer of 1,3-PD was increased. After introducing the PHB synthesis pathway, the final content of acetate was further reduced. At the same time, the engineered strain co-produced 91.2 g/L extracellular 1,3-PD and 2.56 g/L intracellular PHB that could be easily separated from each other, which is very beneficial to the downstream process. The idea developed in this study will be applied in other similar biomanufacturing process for bio-based chemicals production.

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Tables

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	References
Strains		
<i>Klebsiella pneumoniae</i> ATCC 25955	Wild type	ATCC
<i>Escherichia coli</i> S17-1	<i>thi pro hsdR recA Tra</i> ⁺ , conjugative strain able to host λ - <i>pir</i> -dependent plasmids	Kleiner et al., 1988
<i>Escherichia coli</i> DH5 α	Strain for cloning and plasmid propagation	Vazyme
<i>Klebsiella pneumoniae</i> Kpr-1	ATCC 25955 Δ adhE, Δ ldhA	This study
<i>Klebsiella pneumoniae</i> Kpr-2	ATCC 25955 Δ adhE, Δ ldhA and Δ frdA	This study
<i>Klebsiella pneumoniae</i> Kpr-3	Kpr-2 frdA::P _{pc} -acs _{ap}	This study
<i>Klebsiella pneumoniae</i> Kpr-4	Kpr-3 Δ aceA	This study
<i>Klebsiella pneumoniae</i> Kpr-2-acs _{ap}	Kpr-2 harboring pDK7-P _{pc} -acs _{ap}	This study
<i>Klebsiella pneumoniae</i> Kpr-2-acs _{kp}	Kpr-2 harboring pDK7-P _{pc} -acs _{kp}	This study
<i>Klebsiella pneumoniae</i> Kpr-5	Kpr-2 harboring pDK7-phbCBA	This study
<i>Klebsiella pneumoniae</i> Kpr-6	Kpr-4 harboring pDK7-phbCBA	This study
Plasmids		
pKR6K	Cm ^r , gene replacement vector derived from plasmid pK18mobsacB, R6K origin, Mob ⁺ , sacB	Wang et al., 2014
pQKZ103	Amp ^r , pBluescript SK ⁻ , phbCAB genes from <i>R. eutropha</i>	Kang et al., 2008
pDK7	Cm ^r , expressing vector, tac promoter	Simon et al., 1983
pDK7-phbCBA	pDK7 derivative, carries a phbCBA cassette	This study
pDK7-P _{pc} -acs _{ap}	pDK7 derivative, carries a P _{pc} -acs _{ap} cassette	This study
pDK7-P _{pc} -acs _{kp}	pDK7 derivative, carries a P _{pc} -acs _{kp} cassette	This study
pKR6K- Δ adhE	pKR6K derivative, carries a 1837-bp deletion of the	This study

	adhE gene	
pKR6K-ΔldhA	pKR6K derivative, carries a 831-bp deletion of the ldhA gene	This study
pKR6K-ΔfrdA	pKR6K derivative, carries a 1547-bp deletion of the frdA gene	This study
pKR6K-ΔaceA	pKR6K derivative, carries a 1320-bp deletion of the aceA gene	This study
pKR6K-frdA::P _{pc} -acs _{ap}	pKR6K derivative, carries a 1791-bp deletion of the frdA gene and a P _{pc} -acs _{ap} insertion	This study

Amp^r and Cm^r: resistance to ampicillin and chloramphenicol, respectively.

Table 2 Effects of acetyl-CoA synthetase overexpression on metabolite profiles of *K. pneumoniae*

Strains	Lactate (g/L)	Ethanol (g/L)	Succinate (g/L)	1,3-Propanediol (g/L)	2,3-Butanediol (g/L)
Kpr-2	0.26±0.02	0	0.43±0.01	13.92±0.35	6.35±0.24
Kpr-2-P _{pc} -acs _{ap}	0.20±0.01	0.55±0.02	0.75±0.05	12.30±0.43	5.77±0.18
Kpr-2-P _{pc} -acs _{kp}	0.22±0.02	0.59±0.03	0.85±0.04	12.35±0.39	6.17±0.21

The maximum content of ethanol and succinate was obtained after 12 h of shake-flask fermentation. The maximum content of 1,3-propanediol and 2,3-butanediol was obtained after 30 h of shake-flask fermentation. The content of lactate was maintained at a low level.

Figures

Figure 1 Metabolic engineering strategy for 1,3-propanediol production from glycerol in *K. pneumoniae*. The genes shown in red were deleted and the genes shown in blue were overexpressed. Solid lines represent one-step reactions, and dashed lines represent multi-step reactions. 3-HPA, 3-hydroxypropionaldehyde; 3-HB-CoA, 3-hydroxybutyryl-CoA; PHB, polyhydroxybutyrate; *ldhA*, lactate dehydrogenase encoding gene; *adhE*, aldehyde/alcohol dehydrogenase encoding gene; *frdA*, catalytic subunit of fumarate reductase encoding gene; *aceA*, isocitrate lyase encoding gene; *acs*, acetyl-CoA synthetase encoding gene; *phbA*, β -ketothiolase encoding gene; *phbB*, acetoacetyl-CoA reductase encoding gene; *phbC*, PHA synthase encoding gene.

Figure 2 Effects of blocking byproduct pathways on the metabolism of *K. pneumoniae*. (A) Time-course of cell growth; (B) The production of lactate, ethanol and succinate; (C) Time-course of acetate accumulation; (D) The production of 1,3-propanediol and 2,3-butanediol. WT, wild type strain; Kpr-2, the strain with ethanol, lactate, and succinate formation pathway blocked.

Figure 3 Effects of overexpressing acetyl-CoA synthases from different sources. (A) Time-course of cell growth; (B) Time-course of acetate accumulation. Kpr-2, the strain with ethanol, lactate, and succinate formation pathway blocked; Kpr-2-Ppc-acsap, the Kpr-2 strain with expression of heterologous acetyl-CoA synthase from *Acetobacter pastoris* under the constitutive promoter pc; Kpr-2-Ppc-acskp, the Kpr-2 strain with expression of endogenous acetyl-CoA synthase from *K. pneumoniae* under the constitutive promoter pc.

Figure 4 Product profiles of glycerol metabolism in different engineered *K. pneumoniae* under fed-batch fermentation. WT, wild type strain; Kpr-2, the strain with ethanol, lactate, and succinate formation pathway blocked; Kpr-3, Kpr-2 strain with the heterologous acetyl-CoA synthase from *Acetobacter pastoris* under the constitutive promoter pc integrated in the genome; Kpr-6, the Kpr-3 strain with *aceA* gene coding for isocitrate lyase deleted and harboring pDK7-phbCBA for PHB synthesis.

Figure 5 PHB production by the Kpr-6 strain at the end of fermentation with Kpr-2 and Kpr-3 strains as control. Kpr-2, the strain with ethanol, lactate, and succinate formation pathway blocked; Kpr-3, Kpr-2 strain with the heterologous acetyl-CoA synthase from *Acetobacter pastoris* under the constitutive promoter pc integrated in the genome; Kpr-6, the Kpr-3 strain with *aceA* gene coding for isocitrate lyase deleted and harboring pDK7-phbCBA for PHB synthesis.