Klebsiella Pneumoniae Metabolic Regulation and its Application in 1,3-Propanediol Production

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Abstract

Klebsiella pneumoniae is a well-known model organism for glycerol metabolism to produce 1,3-propanediol (1,3-PD), a valuable chemical intermediate for materials, such as polyesters. However, the relatively low conversion rate and productivity, as well as the accumulation of by-products such as lactic acid, ethanol and acetic acid, inhibit the production of 1,3-PD. Hereby, the 1,3-PD metabolism in *K. pneumoniae* was regulated through pathway engineering by using CRISPR-Cas9 technology for the first time to knock out the *ldhA* gene of lactate dehydrogenase,the *adhE* gene of alcohol dehydrogenase and the *ack* gene of acetate kinase respectively as needed and constructed recombinant bacteria IdhA$^{(-)}$, IdhA$^{(-)}$-ack$^{(-)}$, IdhA$^{(-)}$-adhE$^{(-)}$ and IdhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$, all of which showed a decrease in by-product production, leading to a higher NADH availability, and 1,3-BD production was significantly increased. In the shake flask fermentation, the 1,3-PD yield and conversion rate of the recombinant strain IdhA$^{(-)}$, IdhA$^{(-)}$-ack$^{(-)}$, IdhA$^{(-)}$-adhE$^{(-)}$, IdhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ were higher than those of the parent strain. In the fed-batch fermentation, the 1,3-PD yield and conversion rate of the recombinant strain IdhA$^{(-)}$ were higher than those of the parent strain. The biomass of the recombinant strain IdhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ was reduced due to the accumulation of acetic acid, but its 1,3-PD conversion rate was still higher than that of the parent strain. The higher productivity and fewer by-products concluded that the four Klebsiella pneumoniae recombinant strains could be promising industrial strain for economical production of 1,3-PD.

Introduction

Biodiesel fuel is renewable biomass fuel that is used as an alternative fuel of diesel oil. Biodiesel fuel is made from vegetable oils and animal fats by a transesterification reaction or a thermal chemical reaction, in which large amounts of by-product glycerol is produced[1]. With further research and mass production of biodiesel, the production of crude glycerol is also increasing rapidly, leading to new environmental problems. Therefore, new applications of glycerol need to be searched. Nowadays, glycerol can be transformed to many valuable chemicals and one of the promising products obtained is 1,3-propanediol[2].

1,3-PD is a valuable chemical with two hydroxyl groups that has wide applications in the production of lubricants, cosmetics, polymers and pharmaceutical products. For instance, it is used as a precursor for the production of polytrimethylene terephathalate (PTT), which has been commercialized in the textile and carpet industries[3]. Traditionally, most of the production of 1,3-PD has been performed through two chemical synthesis methods. One is acrolein hydration hydrogenation (“DuPont” process), and the other is carbonylation of epoxy ethane (“Shell” process). However there are several drawbacks of these chemical methods. They need strictly controlled conditions with high temperatures and pressures, and require specific catalyst. They also release toxic intermediates and depend on non-renewable materials. And microbial fermentation is relatively an energy-saving and environmental protection industries[4].

Bioconversion of glycerol into 1,3-PD can be efficiently performed by *Klebsiella pneumoniae*[5]. The glycerol is converted into 1,3-PD by a two-step reductive pathway, including glycerol dehydratase (DhaB) catalyzed 3-hydroxypropionaldehyde (3-HPA) synthesis from glycerol and a following reduced nicotinamide adenine dinucleotide (NADH)-dependent 1,3-PD oxidoreductase (DhaT) mediated 1,3-PD formation[6]. The conversion rate of glycerol to 1,3-PD was increased by strain overexpressing DhaB and DhaT[7]. Furthermore, the NAD(+)-dependent gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase (PuuC) was demonstrated to be another hypothetical oxidoreductase for 1,3-PD formation[8]. Nevertheless, considerable amounts of by-product metabolites, especially lactate, acetic acid and ethanol[9], are synthesized via an oxidative pathway with pyruvate as the precursor. Therefore,

elimination of by-product formation is a major strategy to metabolically engineer microorganism to improve 1,3-PD yield[10]. Deletions of genes of ethanol dehydrogenase (*adhE*) for ethanol synthesis,acetate kinase (*ack*) for acetic acid synthesis or lactate dehydrogenase (*ldhA*) for lactate synthesis promoted 1,3-PD production in *K. pneumonia*[11]. In addition, improved NADH regeneration pathway helped recombinant strains to achieve a high productivity[12].

The CRISPR-Cas9 system, as a new type of gene editing technology, can accurately and efficiently knock out or modify the genome, and can be used to obtain gene knockout mutants in a short time[13]. This technology uses an RNA component (sgRNA, single guide RNA) to identify the target site, and uses a single Cas9 protein to cut it to produce double-stranded DNA breaks, thereby achieving efficient gene editing[14]. In addition, CRISPR technology has the ability to knock out multiple genes, or at the same time[15]. Therefore, it can be considered that CRISPR-Cas9 technology may be an ideal choice for blocking the metabolism of by-products.

This study aimed to abolish lactic acid formation through deletion of *ldhA* using the CRISPR-Cas9 technology, and try to block the acetic acid or/and ethanol synthesis pathway based on strains with lactate dehydrogenase gene deleted. By constructing the double gene knockout strains and the three-gene knockout strain, we successfully thereby enhancing enhanced the production intensity of 1,3-PD and reducing the production of by-products.

Materials And Methods

Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this work are listed in Table 1, and the plasmid pattern was shown in Fig. 1a *Escherichia coli* DH5α was used as the host for molecular manipulations. LB medium was used for the routine culture of *E. coli* or *K. pneumoniae.* Ampicillin at concentration of 100 μg mL^{-1} or 50 μg mL^{-1} kanamycin or 30 μg mL^{-1} apramycin was supplemented to the medium when necessary to maintain the plasmids. The temperature for fermentations was 37 °C and flask shake speed was at 220 rpm. The fed-batch fermentations were performed in 1 L bioreactors with 800 μL initial standard medium (2 g L^{-1} yeast extract, 5 mg L^{-1} $FeCl_2$, 0.24 g L^{-1} $MgSO_4$, 3.4 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 1.3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $CaCO_3$, 2 g L^{-1} $(NH_4)_2SO_4$, 20 g L^{-1} glycerol, 1 mL L^{-1} trace elements solutions), respectively. The trace element solution consisted of 70 mg L^{-1} $ZnCl_2$, 0.1 g L^{-1} $MnCl_2{\cdot}4H_2O$, 60 mg L^{-1} H_3BO_3, 0.2 g L^{-1} $CoCl_2{\cdot}2H_2O$, 20 mg L^{-1} $CuCl_2{\cdot}2H_2O$, 25 mg L^{-1} $NiCl_2{\cdot}6H_2O$, 35 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$ and 0.9 mL HCl (37%). In fed-batch fermentation, seventy percent of glycerol was fed into bioreactor to maintain the glycerol concentration between 10 to 20 g L^{-1} during 4 h to 24 h after inoculation, and then the supplement of glycerol was stopped. The broth pH was maintained at 7.0 through the addition of 4 M KOH solution and aeration was set at 0.4 volumes of air per volume of fluid per minute (vvm). The seed cells for the bioreactor were shaken in 500 mL flask containing 200 mL standard medium for 12 h, and subsequently inoculated into the bioreactor at 10% (v/v).

Table 1
Strains and plasmids

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
K. pneumoniae KP	Wild type	Lab. collection
$ldhA^{(-)}$	K. pneumoniae KP ldhA deletion	This work
$ldhA^{(-)}$-$adhE^{(-)}$	$ldhA^{(-)}$ adhE deletion	This work
$ldhA^{(-)}$-$ack^{(-)}$	$ldhA^{(-)}$ ack deletion	This work
$ldhA^{(-)}$-$adhE^{(-)}$-$ack^{(-)}$	$ldhA^{(-)}$-$adhE^{(-)}$ ack deletion	This work
E. coli DH5α		Lab. collection
Plasmids		
pSGKP-km	Kan^r, sgRNA carrier, sucrose sensitive	pSGKP-km was a gift from Quanjiang Ji (Addgene plasmid # 117233;http://n2t.net/addgene:117233; RRID:Addgene_117233)
pCasKP-apr	Apr^r, Carry Cas9 protein, Temperature sensitive	pCasKP-apr was a gift from Quanjiang Ji (Addgene plasmid # 117231;http://n2t.net/addgene:117231; RRID:Addgene_117231)
pSGKP-km-sgRNA	Kan^r, contains each sgRNA corresponding to each gene(*ldhA*, *adhE*, *ack*)	This work

Constructions of plasmids and strains

According to the genome sequence of *K. pneumoniae* KCTC2242, the upstream and downstream sequence of *ldhA* gene (KPN2242_RS10525) was amplified by PCR using the genome of *K. pneumoniae* KP as template with primer: Primer-*ldhA*-SYF, Primer-*ldhA*-SYR, Primer-*ldhA*-XYF and Primer-*ldhA*-XYR. Then, the dsDNA repair template of *ldhA* was synthesized by overlap PCR. By using Micropulser (Bio-Rad, USA) for electroporation, the pCasKP-apr plasmid[16] was transformed into *Klebsiella pneumoniae* KP, and the KP-pCasKP-apr strain was established. Use sgRNAcas9 software to design 3 pairs of *ldhA* spacers sgRNA (sgRNA-*ldhA*-1F, sgRNA-*ldhA*-1R, sgRNA-*ldhA*-2F, sgRNA-*ldhA*-2R, sgRNA-*ldhA*-3F, sgRNA-*ldhA*-3R, then phosphorylate and anneal them, and ligate them with the plasmid pSGKP-km linearized by restriction enzyme *BsaI.* The ligation product is transformed into *E. coli* DH5α and extract the plasmid.That is, the expression vector pSGKP-km-sgRNA has been constructed. The pSGKP-km-sgRNA1-3 plasmids obtained were co-electroporated with the dsDNA repair template to KP-pCasKP-apr competent cells, and *ldhA* was knocked out using the CRISPR-Cas9 double plasmid system. Using Primer-*ldhA*-SYF and Primer-*ldhA*-XYR as primers for colony PCR identification. Select positive colonies with resistance to apramycin and kanamycin, then

cure the two plasmids previously transferred. The successfully constructed *ldhA* deletion recombinant *Klebsiella pneumo*niae was named ldhA$^{(-)}$.

In the same way, refer to the sequence of *adhE* and *ack* in GeneBank (KPN2242_14015, KPN2242_16530) and overlapping PCR primer requirements, design primers: Primer-*adhE*-SYF, Primer-*adhE*-SYR, Primer-*adhE*-XYF, Primer-*adhE*-XYR; Primer-*ack* -SYF, Primer-*ack*-SYR, Primer-*ack*-XYF, Primer-*ack*-XYR. Using *Klebsiella pneumoniae* genome as a template, PCR amplified the upstream and downstream sequences of *adhE* and *ack* respectively. The dsDNA repair templates of *adhE* and *ack* were constructed by overlap PCR respectively. Similarly, the new expression vector pSGKP-*adhE*-sgRNA plasmid and pSGKP-*ack*-sgRNA plasmid also need to be designed and constructed in the manner described in the first paragraph, and the spacer sgRNA designed and used is shown in Table 2. Taking ldhA$^{(-)}$ as the starting strain, the dsDNA repair template was co-electroporated with pSGKP-sgRNA plasmid into the host cell carrying the pCasKP-apr vector. By using the CRISPR-Cas9 double plasmid system, double gene knockout strains and triple gene knockout strains can be constructed. The double gene-deletion bacteria constructed in this experiment include *Klebsiella pneumoniae* with deletion of *ldhA* and *adhE, K. pneumoniae* KP with deletion of *ldhA* and *ack*, and three-gene deletion bacteria including *K. pneumoniae* KP with deletion of *ldhA, adhE* and *ack*. After curing plasmid, the strains verified to be correct after PCR and genome sequencing were named ldhA$^{(-)}$-adhE$^{(-)}$, ldhA$^{(-)}$-ack$^{(-)}$ and ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ respectively.

Table 2
The spacer sgRNA used to construct plasmid pSGKP-*adhE*-sgRNA and plasmid pSGKP-*ack*-sgRNA

Spacer	sequence	application
ldhA-SYF	TCGCTTCCGCCAGCC	upstream sequence of *ldhA* gene
ldhA-SYR	GAGCACAAAAGGGAAAGGCAAAGACTTTTCTCCAGTGATTATACCGTCAC	
ldhA-XYF	AATCACTGGAGAAAAGTCTTTGCCTTTCCCTTTTGTGCTCC	downstream sequence of *ldhA* gene
ldhA-XYR	GTTTTCCGTCAGATCGACCTGC-3	
adhE-SYF	CGAACAGTGATTGAGTCACATTGGG	upstream sequence of *adhE* gene
adhE-SYR	CTTGTCGCGATGCTATCGCTGTTGGGTAAATCAGTCGCTCGATTCGACATTATAG	
adhE-XYF	CTATAATGTCGAATCGAGCGACTGATTTACCCAACAGCGATAGCATCGCGACAAG	downstream sequence of *adhE* gene
adhE-XYR	GCCAGTTTCTGACGCGTGA	
ack-SYF	CGATTGGCGGCATGATGC	upstream sequence of *ack* gene
ack-SYR	CGAATTATTGCGGGTCAGGGCTGGCCGTCAGTGTGTCATTAGCGTAGCGC	
ack-XYF	GCGCTACGCTAATGACACACTGACGGCCAGCCCTGACCCGCAATAATTCG	downstream sequence of *ack* gene
ack-XYR	CTGAGTATTCGCGTGGTAGTTGG-3	
sgRNA-*ldhA*-1F	TAGTCAGCACGTTAATGATGCATA	Guide RNA (sgRNA) for *ldhA* gene knockout
sgRNA-*ldhA*-1R	AAACTATGCATCATTAACGTGCTG	
sgRNA-*ldhA*-2F	TAGTGATGCCAATTTCTCCCTCGA	
sgRNA-*ldhA*-2R	AAACTCGAGGGAGAAATTGGCATC	
sgRNA-*ldhA*-3F	TAGTGTACGTTATGGCAGGCGGAG	
sgRNA-*ldhA*-3R	AAACCTCCGCCTGCCATAACGTAC	
sgRNA-*adhE*-1F	TAGTACTCGCGCCTTCCCTGACTC	Guide RNA (sgRNA) for *adhE* gene knockout
sgRNA-*adhE*-1R	AAACGAGTCAGGGAAGGCGCGAGT	
sgRNA-*adhE*-2F	TAGTTGCGGAGAGGCCCAGGTGAT	
sgRNA-*adhE*-2R	AAACATCACCTGGGCCTCTCCGCA	
sgRNA-*adhE*-3F	TAGTTTCTGAGTTCTCTGACGGCC	
sgRNA-*adhE*-3R	AAACGGCCGTCAGAGAACTCAGAA	
sgRNA-*ack*-1F	TAGTAGCAAACAAGAAGCCGAGTT	Guide RNA (sgRNA) for *ack* gene knockout
sgRNA-*ack*-1R	AAACAACTCGGCTTCTTGTTTGCT	
sgRNA-*ack*-2F	TAGTATCGACGAGTCCGTAATTCA	
sgRNA-*ack*-2R	AAACTGAATTACGGACTCGTCGAT	
sgRNA-*ack*-3F	TAGTGAGATAGGATTCTTCCGGCA	
sgRNA-*ack*-3R	AAACTGCCGGAAGAATCCTATCTC	

Assay of biomass and metabolites

The biomass concentration was measured using the absorbance of the supernatant at 600 nm using an Agilent UV/VIS spectrophotometer.

The metabolites of glycerol, 1,3-propanediol, lactate, ethanol, acetic acid and 2,3-butanediol in broths during fermentation were analyzed with a SHIMAZU 10AVP HPLC system with a refractive index detector. A Bio-Rad Amines HPX-87H organic acids column was used with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1} and a column temperature at 65 ^oC. Samples were microfiltered through 0.45 μm membranes prior to analysis.

Results

Construction of the recombinant strains

The expression vector pCasKP-apr plasmid was transformed into *K. pneumoniae* by electrotransformation, and the resistant monoclonal KP-pCasKP-apr can be obtained on the plate. The plasmid pCasKP-apr was used as a positive control, the original strain of *Klebsiella pneumoniae* was used as a negative control, and pCaskp-*ldhA*-F(5'-GAAATGCGGCTGGTGCG-3') and pCaskp-*ldhA*-R(5'-CGGTTTGGTTAGCGAGAAGAG-3') were used as primers to perform a positive single clone colony PCR. Fig. S6 shows that the sample and the positive control band are consistent, indicating that the plasmid has been successfully transferred into the host cell K. pneumoniae. Fig. S7 shows the upstream and downstream sequences of *ldhA*, *adhE* and *ack* amplified by PCR. Then the upstream and downstream sequences of *ldhA*, *adhE* and *ack* were annealed by overlap PCR to synthesize the dsDNA repair templates (Fig. S8). Fig. S9 shows the constructed pSGKP-km-sgRNA plasmid of each target gene. They were identified by PCR with the corresponding Spacer-F and M13R as primers, and bright bands appeared at 200–300 bp, which were consistent with expectations.

The constructed pSGKP-km-sgRNA plasmid and the dsDNA repair template were co-electroporated into competent cells carrying the plasmid pCasKP-apr, and the electro-transformed host was cultured on the screening double-resistance LB agar plate to obtain resistant individual colonies (Fig. 1b-d). After curing the pCasKP-apr and pSGKP-km plasmids, The 1 kb fragment was amplified from the chromosome DNA of the $ldhA^{(-)}$, while 2 kb DNA fragment was obtained from *K. pneumoniae* KP (Fig. S10), indicating the *ldhA* gene has been successfully deleted from *K. pneumoniae* KP. Similarly, the chromosomal DNA from $ldhA^{(-)}$-$adhE^{(-)}$ and $ldhA^{(-)}$-$ack^{(-)}$ were also amplified with a 1 kb fragment, which are in contrast with about 2 kb DNA fragment obtained from *Klebsiella pneumoniae* KP (Fig. S11). This indicates that the *adhE* and *ack* genes have been successfully knocked out from $ldhA^{(-)}$, and means the double gene deletion strain of *Klebsiella pneumoniae* was constructed, which were named $ldhA^{(-)}$-$adhE^{(-)}$ and $ldhA^{(-)}$-$ack^{(-)}$, respectively. Through this CRISPR-Cas9 double-plasmid system gene editing method, *Klebsiella pneumoniae* bacteria with all the three genes of *ldhA, adhE* and *ack* deleted were also successfully constructed (Fig. 1e, Fig. S11), and it was named $ldhA^{(-)}$-$adhE^{(-)}$-$ack^{(-)}$.

Effects of ldhA, adhE **and** ack inactivation on metabolism of K. pneumoniae

To investigate the effects of *ldhA, adhE* and *ack* inactivation on metabolism of *K. pneumoniae*, batch cultures of the wild type strain and recombinant strains were performed in shake flasks with the initial glycerol concentration at 20 g L^{-1}. The cell growth, glycerol consumption and production of main and by-products of metabolism were monitored.

As shown in Fig. 2 and Table 3, the biomass of the recombinant strain IdhA$^{(-)}$ was significantly higher than that of the original strain of *Klebsiella pneumoniae* KP, reaching the maximum at 12h, with an OD_{600} of 6.96. The original bacteria also reached the maximum at 12h, with an OD_{600} of 5.06. The biomass of IdhA$^{(-)}$ increased by 37.5% compared with the original *Klebsiella pneumoniae* KP. In addition, the delay period of IdhA$^{(-)}$ was very short, indicating that it can quickly adapt to the new growth environment. The metabolism of the recombinant strain IdhA$^{(-)}$ has also undergone significant changes. The genetically engineered strain no longer produces lactic acid, and the production of 1,3-propanediol has also been increased. Compared with the original strain, the 1,3-PD concentration and conversion rate increased by 56.7% and 56.5%, respectively. At the same time, the concentration of 2,3-butanediol and ethanol also increased slightly. This may be due to reduced competition for intracellular NADHs by by-products and improvement in all NADH-dependent metabolic pathways.

Table 3

Comparison of final metabolites concentration of recombinant IdhA$^{(-)}$ with those of wild type *K. pneumoniae* KP in batch fermentations

Strains	Products concentration (g L^{-1})			1,3-PD conversion (mol mol^{-1})
	1,3-PD	Lac	2,3-BD	
K.pneumoniae KP	3.88	0.08	4.32	0.23
IdhA$^{(-)}$	6.08	0	3.52	0.36

Figure 3 and Table 4 show the comparison of double and triple gene deletions recombinant strains relative to the original strains of *Klebsiella pneumoniae* KP by shaker fermentation. The biomass and growth trend of the recombinant strain IdhA$^{(-)}$-ack$^{(-)}$ is higher than that of *Klebsiella pneumoniae* KP, and it's biomass increased by 7.07% compared with that of the original bacteria KP. At the same time, the original bacteria showed a downward trend after 12 hours, but the recombinant bacteria continued to grow, indicating that after both the lactic acid and acetic acid metabolic pathways were blocked, cell growth was not harmed. However, the highest biomass of IdhA$^{(-)}$-adhE$^{(-)}$ was 13.52% lower than that of the original bacteria, indicating that the simultaneous deletion of *IdhA* and *adhE* had a certain negative effect on cell growth. Moreover, the increase of the byproduct acetic acid yield may be one of the factors affecting the growth of the bacteria. Compared with the original strain, the biomass of the recombinant strain IdhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ had no significant change. The results showed that after deleting the three genes using the CRISPR-Cas9 double plasmid system, the cells were still not lethal or significantly inhibited.

Table 4

Comparison of final metabolites concentration of recombinant IdhA$^{(-)}$-adhE$^{(-)}$, IdhA$^{(-)}$-ack$^{(-)}$ and IdhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ with those of wild type K. pneumoniae **KP in batch fermentations**

Strains	Products concentration (g L^{-1})				1,3-PD conversion (mol mol^{-1})
	1,3-PD	Ace	Eth	2,3-BD	
K.pneumoniae KP	6.04	0.22	0.41	1.68	0.37
IdhA$^{(-)}$-adhE$^{(-)}$	7.75	0.37	0.34	2.81	0.47
IdhA$^{(-)}$-ack $^{(-)}$	6.91	0.19	0.38	2.08	0.42
IdhA$^{(-)}$-adhE$^{(-)}$-ack $^{(-)}$	8.29	0.27	0.37	2.63	0.50

In addition, compared with the original strain, the concentration of 1,3-PD, the conversion rate, and the concentration of 2,3-BD of the recombinant strain ldhA$^{(-)}$-ack$^{(-)}$ increased by 14.4%, 13.51% and 23.81%. In addition, it no longer produced acetic acid in the first 8h of the fermentation process on the basis of producing no lactic acid. And the concentration of ethanol was also 35.67% lower than that of the original strain *K. pneumoniae* KP. At the same time, compared with the original strain *K. pneumoniae* KP, the byproduct ethanol production of ldhA$^{(-)}$-adhE$^{(-)}$, decreased significantly, by 62.23%. Furthermore, inactivation of the key enzyme for ethanol formation lead to the increased glycerol flux to 1,3-PD[17]. The concentration of 1,3-PD (7.75 g/L) and its conversion (0.47 mol/mol) were increased by 28.39% and 27.03%, respectively. However, the output of the by-product acetic acid has increased significantly, which inhibited the growth of the bacteria. It may need to be solved by improving the acid resistance of the strain. For the recombinant strain ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$, on the basis of not producing lactic acid, compared with the original strain *K. pneumoniae* KP, the output of ethanol was greatly reduced by 78.5%. But the acetic acid content was 22.73% higher than that of the original strain. According to the observation in Fig. 3, it is speculated that the cells may have converted to consume 2,3-BD as a substrate and produce acetic acid through other metabolic pathways. The 1,3-PD concentration and conversion rate of recombinant ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ increased by 37.31% and 37.53%, respectively, compared with KP. And the concentration of 2,3-BD, compared with *K. pneumoniae* KP, increased by 56.97%. In summary, the above results show that blocking the two or three by-product synthesis pathways at the same time facilitates the flow of reduced NADH to the main product synthesis pathway, effectively promotes the generation and accumulation of the main product, and also simplifies the downstream separation of the main product.

Fed-batch fermentation and metabolic flux redistribution

As results described above, the recombinant ldhA$^{(-)}$ and ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ both exhibited great ability of cell growth and 1,3-PD production. The further investigation of metabolic flux redistribution in fed-batch fermentation was performed in 1 L fermentor using wild type *K. pneumoniae* KP as the control. Concentration of major metabolites (1,3-PD, 2,3-butanediol, lactate, ethanol and acetate) were determined. The fermentation results of ldhA$^{(-)}$ were shown in Fig. 4 and Table 5. And the fermentation results of ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ were shown in Fig. 5 and Table 6.

Table 5

Comparison of final metabolites concentration of recombinant ldhA$^{(-)}$ with those of wild type *K. pneumoniae* KP in fed-batch fermentations

Strains	Products concentration (g L^{-1})				Glycerin consumption (g)	1,3-PD conversion (mol mol^{-1})
	1,3-PD	Ace	Lac	2,3-BD		
K.pneumoniae KP	36.62	1.72	0.41	4.10	103.3	0.34
ldhA$^{(-)}$	40.52	1.76	0	7.45	102.57	0.38

Table 6

Comparison of final metabolites concentration of recombinant ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ with those of wild type *K. pneumoniae* KP in fed-batch fermentations

Strains	Products concentration (g L^{-1})					Glycerin consumption (g)	1,3-PD conversion (mol mol^{-1})
	1,3-PD	Ace	Lac	Eth	2,3-BD		
K.pneumoniae KP	36.39	0.82	0.40	0.19	8.61	95.55	0.36
ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$	30.71	1.20	0	0	8.48	66.17	0.43

It can be seen from Fig. 4 and Table 5 that the highest biomass OD_{600} (10.74) of the recombinant strain ldhA$^{(-)}$ was 7.82% lower than the OD_{600} (11.58) of the original strain. However, the growth rate of recombinant bacteria was increased, indicating that it had good growth performance, and the deletion of the *ldhA* gene did not cause significant inhibition of the cells. With 24 h as the end point of fermentation, the recombinant bacteria consumed 102.57 g glycerol and produced 40.52 g/L 1,3-PD and 7.45 g/L 2,3-BD. Compared with the original strain *K. pneumoniae*, the concentration and conversion rate of 1,3-PD and the concentration of 2,3-BD were increased by 10.65%, 11.77% and 81.71%, respectively. In addition, the recombinant bacteria ldhA$^{(-)}$ no longer produced by-product lactic acid, and the production level of acetic acid was basically the same as that of the original bacteria. The above results indicate that blocking the lactic acid synthesis pathway effectively reduces the competition for reducing power, so that more NADH flows to the synthesis pathways of 1,3-PD and 2,3-BD.

It can be seen from Fig. 5 and Table 6 that the biomass OD600 (7.79) of the recombinant strain ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ *was* lower than the OD600 (11.56) of the original strain *K. pneumoniae* KP. The maximum biomass of recombinant bacteria was even nearly 4 OD less than that of wild-type bacteria. This indicated that the growth of the bacteria was significantly inhibited after the deletion of the three genes. The above results may be caused by the following reasons: First, the increase of acetic acid production inhibited cell growth; Secondly, the deletion of multiple genes may increase the lethality of the strain. The third is that the massive reduction of by-products leads to the disturbance of the reducing power of NADH and NAD + which maintains the redox balance in the cell. Compared with the original strain *K. pneumoniae*, the recombinant strain ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ no longer produceed by-products of lactic acid and ethanol, and glycerol fluxed to the main products 1,3-PD and 2,3-BD increased accordingly. Since the growth of ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ was inhibited by the increase of acetic acid concentration, the decrease in biomass reduced the production of 1,3-PD by 18.5%. However, due to the redistribution of metabolic flux, the conversion rate of 1,3-PD (0.43 mol/mol) was somewhat higher than that of the original strain (0.36 mol/mol), which increased by 19.44%. The results show that ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ with few by-products and high 1,3-propanediol conversion rate has a certain potential for large-scale production of major products.

Discussion

1,3-Propanediol is a high-value chemical raw material. The use of microbial methods for material synthesis has the characteristics of mild reaction conditions, high conversion rate, low equipment investment, easy operation and environmental friendliness. Among the natural production strains of 1,3-propanediol, the metabolic pathway of Klebsiella pneumoniae is relatively clear, and the biochemical properties and genetic background of Escherichia coli are very similar, so it has received more attention. However, during the fermentation of Klebsiella pneumoniae to produce 1,3-propanediol, by-products such as lactic acid, acetic acid and ethanol are produced, which harm the growth of bacteria to a certain extent. The production of by-products also robs the reducing power of the main product synthesis pathway and leads to a decrease in the yield of 1,3-PD. In addition, the accumulation of by-products affects the post-extraction and purification of 1,3-PD, which increases the difficulty of downstream processing and production costs. At present, the existing literature reports that the regulatory targets are relatively concentrated and single, and only the local metabolism of the 1.3-propanediol metabolic pathway has been modified. More research work has focused on the use of traditional techniques such as mutagenesis and λRed recombination to modify the genome to develop new strains, but traditional gene editing techniques have shortcomings. Among various gene editing methods, CRISPR-Cas9 technology, as a new type of gene editing technology, is more precise and efficient. Therefore, this study is beneficial to simplify the subsequent extraction and purification, and reduce the difficulty and production cost of downstream processing; at the same time, it provides a certain theoretical basis and application guidance for the promotion of microbial production of 1,3-propanediol.

Conclusions

The 1,3-PD metabolism is controlled by the NADH availability. This work enhanced the bio-based production of 1,3-PD by deleting the key enzyme genes in the main by-product synthesis pathway, and enhanced NADH availability. The novel constructed recombinant ldhA$^{(-)}$,nldhA$^{(-)}$-adhE$^{(-)}$, ldhA$^{(-)}$-ack$^{(-)}$ and ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ exhibited great 1,3-PD productivity without any lactate accumulation. The other by-products are correspondingly greatly reduced, and the strains has strong genetic stability. However, the three-gene deletion recombinant bacterium ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ has higher acetic acid content. And how to reduce the output of acetic acid is the key research direction in the future. The comprehensive conclusion is that the recombinant ldhA$^{(-)}$, ldhA$^{(-)}$-adhE$^{(-)}$, ldhA$^{(-)}$-ack$^{(-)}$ and ldhA$^{(-)}$-adhE$^{(-)}$-ack$^{(-)}$ could be used as promising producers for efficient and economical industrial production of 1,3-PD.

Declarations

Associated Content

Author Contributions

F.H.W and Y.D.W conceived the project. Y.D.W, J.M, Y.H.L, Y.X.W, X.Y and B.X performed the experiment and analyzed the data. Y.D.W and F.H.W. wrote the manuscript. All authors discussed the results and revised the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of data and material

The authors declare that all data can be shared and accessed by the permission of the journal publication rules. The data underlying this article are available in the article and in its online supplementary material.

Ethics Approval

Not applicable

Consent to Participate

All participants gave their consent to participate.

Consent for Publication

All authors have their consent to publish their work.

Code availability

Not applicable

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Figures

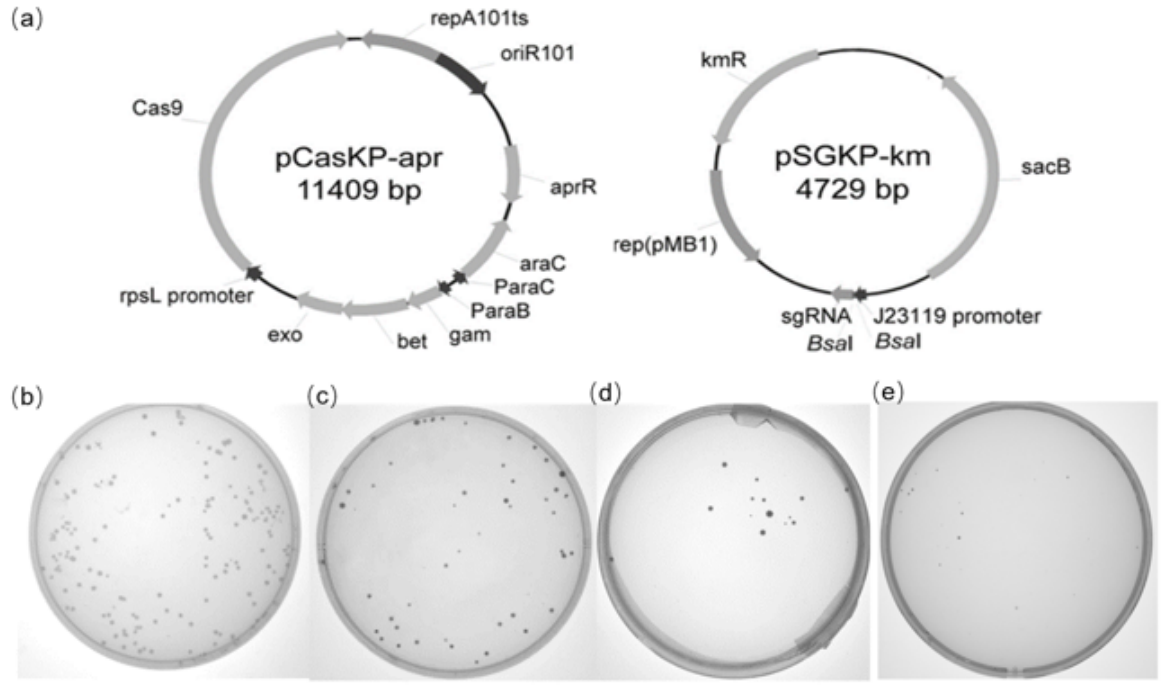

Figure 1

Plasmid profiles and Recombinant Klebsiella pneumoniae with target gene deletion

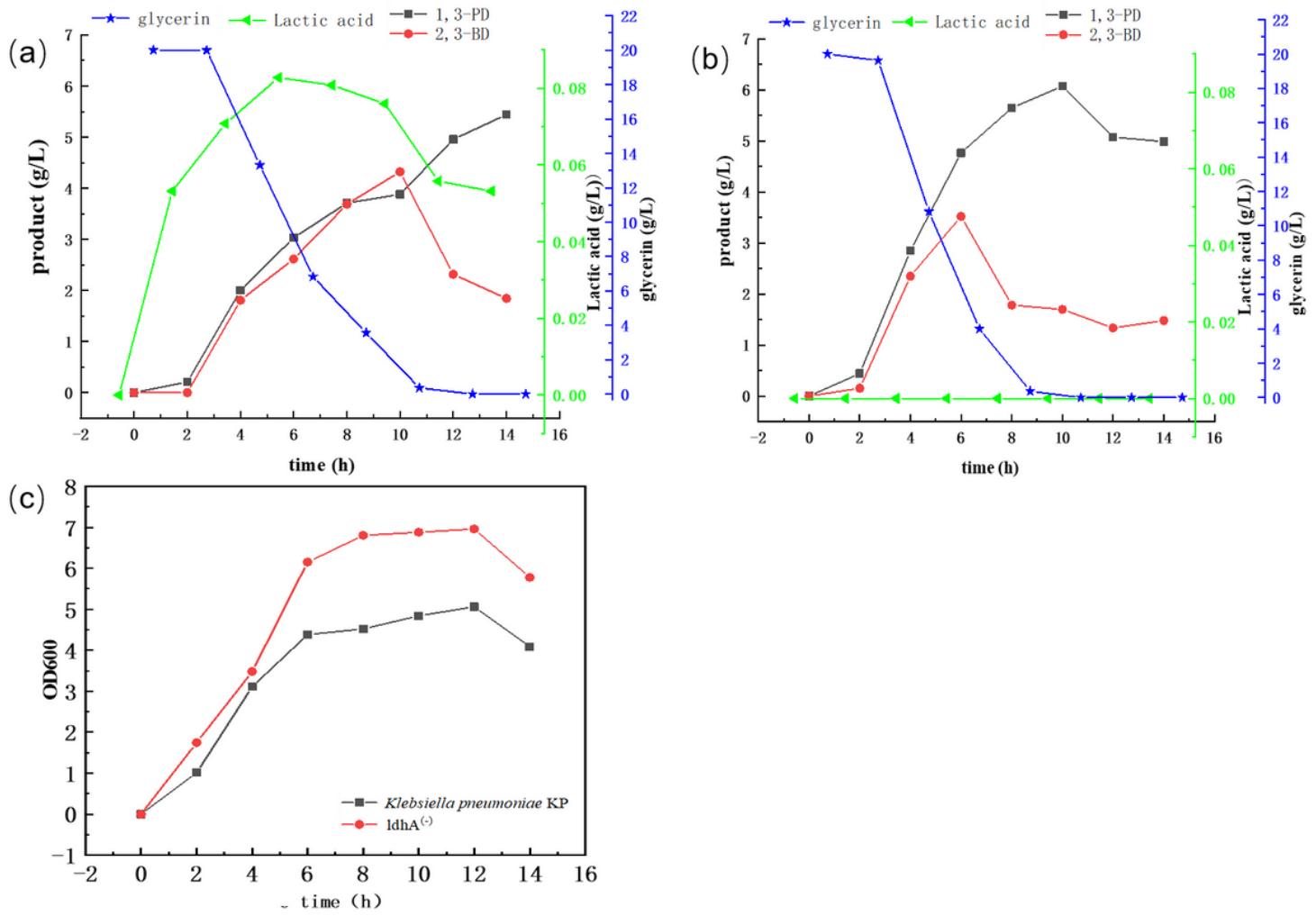

Figure 2

Batch culture of wild type and recombinant strains ldhA(-)

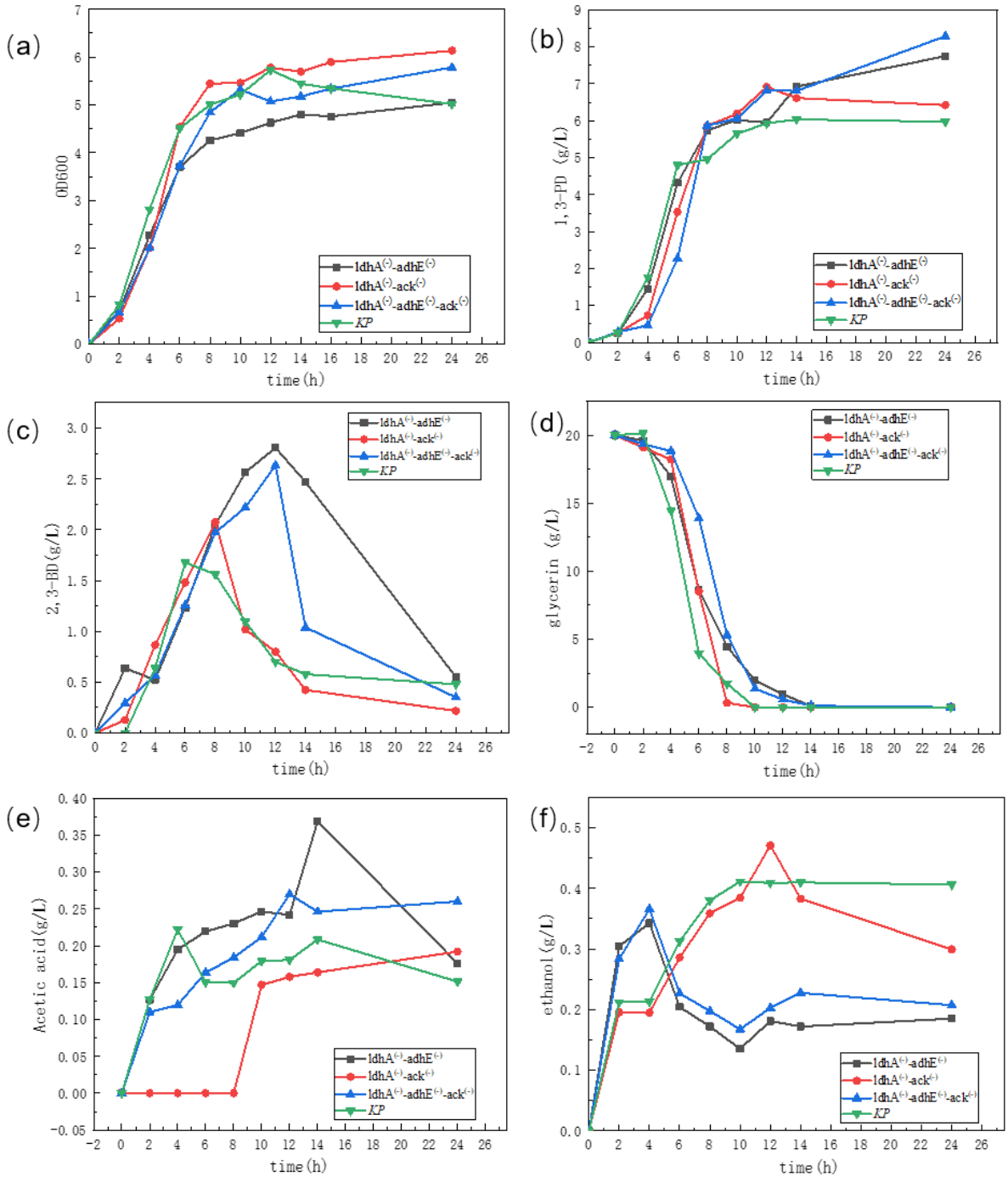

Figure 3

Batch culture of wild type and recombinant strains ldhA(-)-adhE(-), ldhA(-)-ack(-) and ldhA(-)-adhE(-)-ack (-)

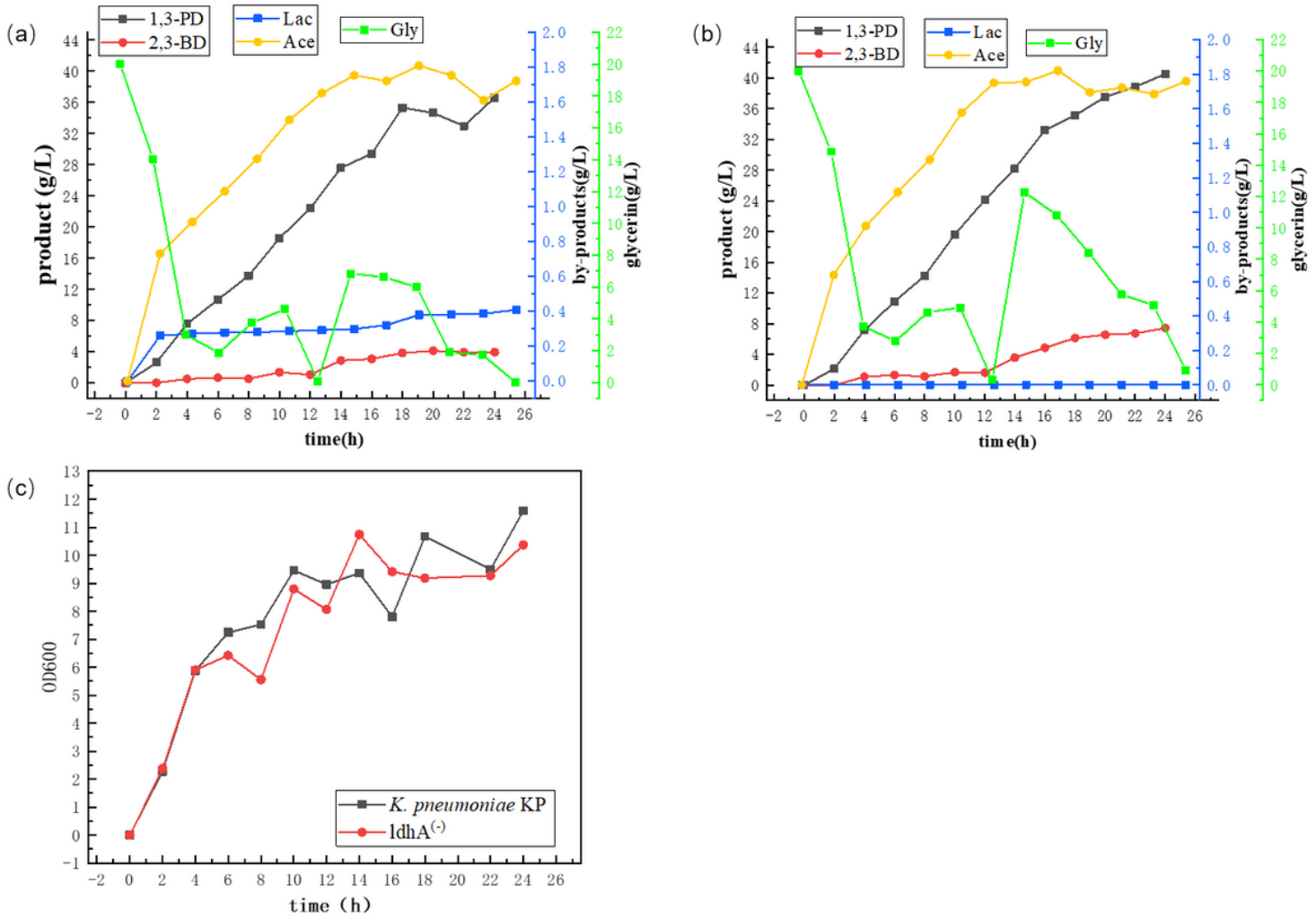

Figure 4

Fed-batch fermentation of the wild type K. pneumoniae KP and the recombinant IdhA(-) in 1 L bioreactors

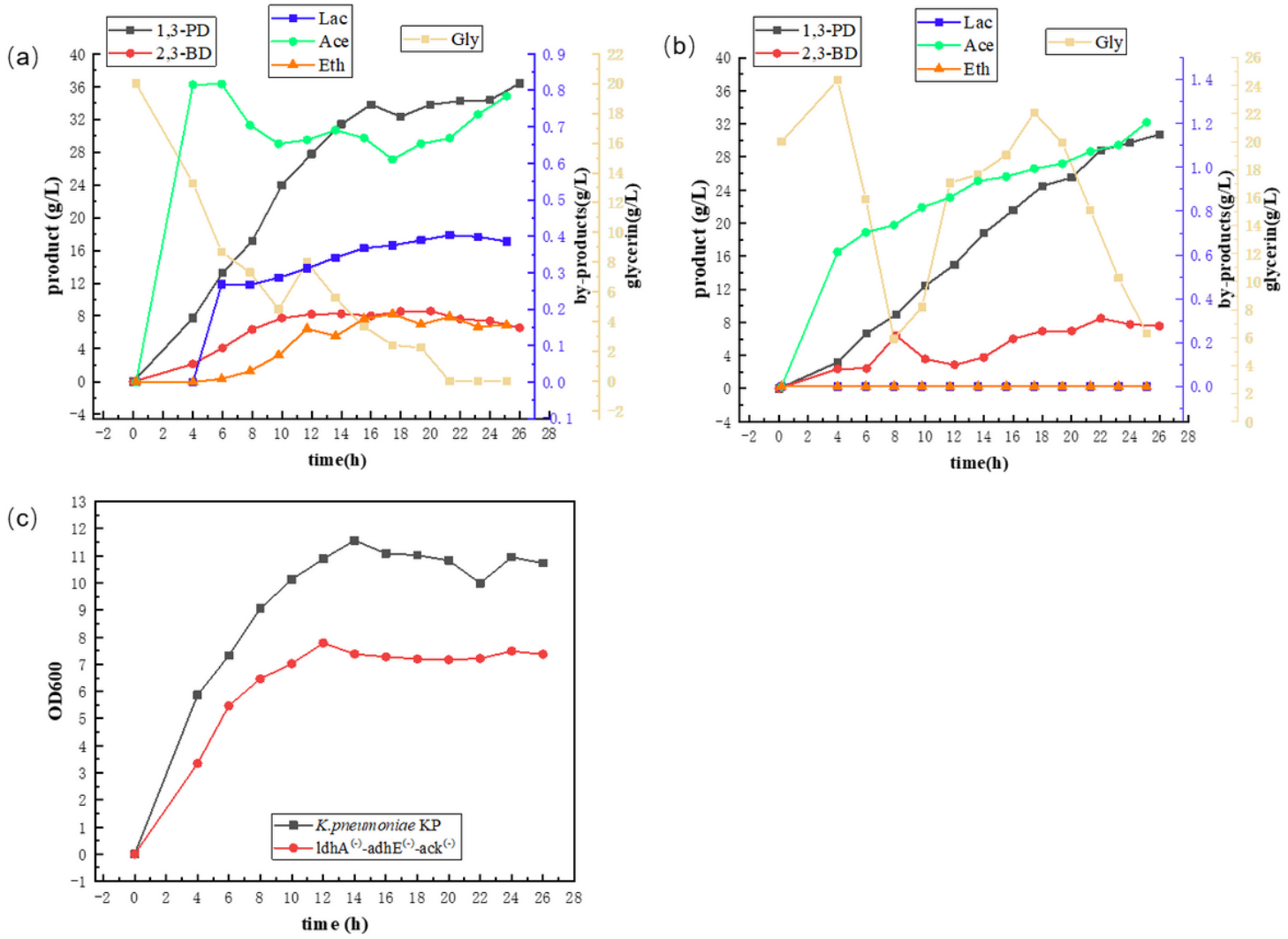

Figure 5

Fed-batch fermentation of the wild type K. pneumoniae KP and the recombinant ldhA(-)-adhE(-)-ack(-) in 1 L bioreactors

Supplementary Files

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