RSC Advances

PAPER

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Cite this: *RSC Adv.*, 2014, **4**, 57791

1-Butanol production from glycerol by engineered *Klebsiella pneumoniae*

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Received 27th August 2014
Accepted 20th October 2014

DOI: 10.1039/c4ra09016k

www.rsc.org/advances

To utilize the by-product of biodiesel production, *Klebsiella pneumoniae*, a well-known glycerol-fermenting microorganism, was engineered to produce 1-butanol. The modified CoA-dependent and 2-keto acid pathways were established by expressing the genes *ter-bdhB-bdhA* and *kivd*, respectively. The 1-butanol titer and specific BuOH yield were 15.03 mg L^{-1} and 27.79 mg-BuOH per g cell in *KpTBB* (*K. pneumoniae* overexpressing the genes *ter-bdhB-bdhA*), and 28.7 mg L^{-1} and 51.58 mg-BuOH per g cell in *Kp-kivd* (*K. pneumoniae* overexpressing the gene *kivd*), respectively. Moreover, the native products in *K. pneumoniae* fermentation were down regulated using the antisense RNA strategy. The resulting yield of 1,3-propanediol and 2,3-butanediol was reduced by 81% and 15%, respectively. This work reports a new strain, *K. pneumoniae*, for 1-butanol production and the application of an antisense RNA strategy as an effective method for reducing the main by-products.

1. Introduction

Alternative energy sources have gained increasing popularity due to the shortage of fuel and the increasing global environmental concerns. Over the past few decades, ethanol has been regarded as a major renewable substitute of fuel.[1,2] Ethanol, however, is not ideally suited because of its lower energy density and hygroscopicity-resulting storage and distribution problems.[3–5] In addition, due to the limits set by regulations and the requirements for engine modification, the percentage of ethanol blended with gasoline is generally limited to 10%. Compared to ethanol, 1-butanol has higher energy density and lower vapor pressure, and can be blended with gasoline up to 85% and used to fuel cars without engine modification.[6] Therefore, 1-butanol, especially biobutanol, is considered a highly promising alternative fuel.[6–8]

The microbial production of 1-butanol using lower cost substrates has received worldwide attention, and *Clostridium* species, a solventogenic bacteria, can produce 1-butanol by anaerobic fermentation.[9,10] On the other hand, the industrial production of 1-butanol by *Clostridium* species has been restricted due to its complex physiology and difficulty for genetic modification.[9–11] Many other engineered strains have been used for 1-butanol production. *E. coli*, which has a higher growth rate than *Clostridium* species,[12] was engineered to produce 1-butanol by transferring the CoA-dependent 1-butanol production pathway[9] or by introducing the synthetic 2-keto acid pathway.[3,10] *Saccharomyces cerevisiae*, which has a higher tolerance to ethanol and potentially to 1-butanol than *Clostridium* species,[12] was engineered with some substituted *Clostridium* enzymes for 1-butanol production.[13] *Clostridium tyrobutyricum*,[14] a native butyric acid-producing bacterium, was introduced the aldehyde/alcohol dehydrogenase of *Clostridium acetobutylicum* to produce 1-butanol. *Lactobacillus brevis*,[15] which has a higher tolerance to 1-butanol, was engineered to utilize C5 and C6 substrates to produce 1-butanol. *Synechococcus elongatus*,[16] a kind of autotrophic photosynthetic microorganism, was engineered by introducing a CoA-dependent pathway to produce 1-butanol utilizing CO_2. The byproducts[10,17] in host fermentation, however, are normally reduced by RED system or homologous recombination. Although they are suitable for knocking out a single gene, they are not practical for more advanced metabolic engineering strategies according to the introduced multi FRT sites or the multi selection markers. The asRNA strategy[18] could be an alternative and possibly a more flexible and empowering method to down regulate the enzyme level and product titer.

Glycerol, as a by-product of biodiesel production, is produced abundantly every year.[19,20] Utilizing the crude glycerol and converting it to value-added products have become increasingly attractive.[21,22] *Klebsiella pneumoniae*, which can grow on glycerol as the sole carbon source, has been studied extensively since the 1980s. Although *K. pneumoniae* is a pathogenic microorganism, a great deal of research on chemicals (1,3-propanediol,[17,19] 3-hydroxypropionic acid,[23,24] 2-butanol[25]) production has been conducted. This is due mainly to its capacity to metabolize glycerol and having a similar genetic background to *Escherichia coli*, together with rapid cell proliferation.[23] To our knowledge, *K. pneumoniae* has not been engineered to produce 1-butanol.

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In this study, we established two kinds of 1-butanol synthesis pathways to convert glycerol to 1-butanol in engineered *K. pneumoniae*. In contrast, the main by-products, 1,3-propanediol (1,3-PDO) and 2,3-butanediol (2,3-BD), were down regulated by antisense RNA (asRNA) strategy.

2. Materials and methods

2.1. Reagents

Restriction enzymes and ligase were obtained from New England Bio-labs (Ipswich, MA). Polymerase and other enzymes were obtained from TAKARA (Dalian, China). The kits used for genomic DNA isolation, plasmid extraction and gene retrieve were purchased from OMEGA (America). The DNA recovery kit was purchased from Biomed (Beijing, China). All other chemicals used in this study were of analytical grade or chromatographic grade from Beijing Chemical Company (Beijing, China).

2.2. Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. The fundamental vectors pET-pk and pACYC-pk were devised from pET-28a and pACYC-Duet by substituting the original promoter with a native promoter *pk* of the first subunit of the *dhaB* gene cluster (Genebank no. U30903) in *K. pneumoniae*. The construction strategies for all plasmids and strains in Table 1 are shown in Fig. 1.

All plasmids listed in Table 1 were sequenced to verify the cloning accuracy. All oligonucleotides are listed in Table 2.

2.3. Culture media, inoculation and flask culture of recombinant *K. pneumoniae*

E. coli TOP10 and the derivatives were grown in LB medium (5 g yeast extract, 5 g NaCl, and 10 g tryptone per liter water). The seed culture of *K. pneumoniae* was LB with a relevant antibiotic. The flask culture of *K. pneumoniae* and the derivatives were *Kp*

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[b]	Reference or source
Strains		
E. coli TOP10	Applied for harvesting plasmid	Lab. Collection
Klebsiella pneumoniae	Wild-type	Lab. Collection
KpTBB	pET-TBB	This study
Kp-kivd	pET-kivd	This study
KpTBB-dhaB1	pET-TBB, pACYC-dhaB1	This study
KpTBB-dhaB2	pET-TBB, pACYC-*dhaB2*	This study
KpTBB-dhaB3	pET-TBB, pACYC-*dhaB3*	This study
KpTBB-gdrf1	pET-TBB, pACYC-*gdrf1*	This study
KpTBB-gdrf2	pET-TBB, pACYC-*gdrf2*	This study
KpTBB-dhaT	pET-TBB, pACYC-*dhaT*	This study
KpTBB-gdrf12	pET-TBB, pACYC-*gdrf12*	This study
KpTBB-dhaB123	pET-TBB, pACYC-*dhaB123*	This study
KpTBB-gdrf12-dhaB123	pET-TBB, pACYC-gdrf12-dhaB123	This study
KpTBB-gdrf12-dhaB123-dhaT	pET-TBB, pACYC-gdrf12-dhaB123-dhaT	This study
KpTBB-alaS	pET-TBB, pACYC-*alaS*	This study
KpTBB-budC	pET-TBB, pACYC-*budC*	This study
Plasmids		
pET28a	pBR322 ori, Kana^r	This study
pET-pk	pBR322 ori, Kana^r, pk promoter	This study
pET-TBB	From pET-pk, Ppk::*ter*(TD)-Ppk::*bdhB*(CA)-Ppk::*bdhA*(CA)	This study
pET-kivd	From pET-pk, Ppk::*kivd*(LL)	This study
pACYC-Duet	P15A ori, Cm^r	
pACYC-pk	p15A ori, Cm^r, pk promoter	This study
pACYC-bdhB1	p15A ori, Cm^r, Ppk::*dhaB1*^{a}	This study
pACYC-bdhB2	p15A ori, Cm^r, Ppk::*dhaB2*^{a}	This study
pACYC-bdhB3	p15A ori, Cm^r, Ppk::*dhaB3*^{a}	This study
pACYC-gdrf1	p15A ori, Cm^r, Ppk::*gdrf1*^{a}	This study
pACYC-gdrf2	p15A ori, Cm^r, Ppk::*gdrf2*^{a}	This study
pACYC-dhaT	p15A ori, Cm^r, Ppk::*dhaT*^{a}	This study
pACYC-gdrf12	p15A ori, Cm^r, Ppk::*gdrf2*^{a}-*gdrf1*^{a}	This study
pACYC-dhaB123	p15A ori, Cm^r, Ppk::*dhaB3*^{a}-*dhaB1*^{a}-*dhaB2*^{a}	This study
pACYC-gdrf12-dhaB123	p15A ori, Cm^r, Ppk::*gdrf2*^{a}-*gdrf1*^{a}-*dhaB3*^{a}-*dhaB1*^{a}-*dhaB2*^{a}	This study
pACYC-gdrf12-dhaB123-dhaT	p15A ori, Cm^r, Ppk::*gdrf2*^{a}-*gdrf1*^{a}-*dhaB3*^{a}-*dhaB1*^{a}-*dhaB2*^{a}-*dhaT*^{a}	This study
pACYC-alaS	p15A ori, Cm^r, Ppk::*alaS*^{a}	This study
pACYC-budC	p15A ori, Cm^r, Ppk::*budC*^{a}	This study

[a] A 300 bp reverse complementary sequence of the subject gene. [b] Kana^r, Kanamycin resistance; Cm^r, chloramphenicol resistance.

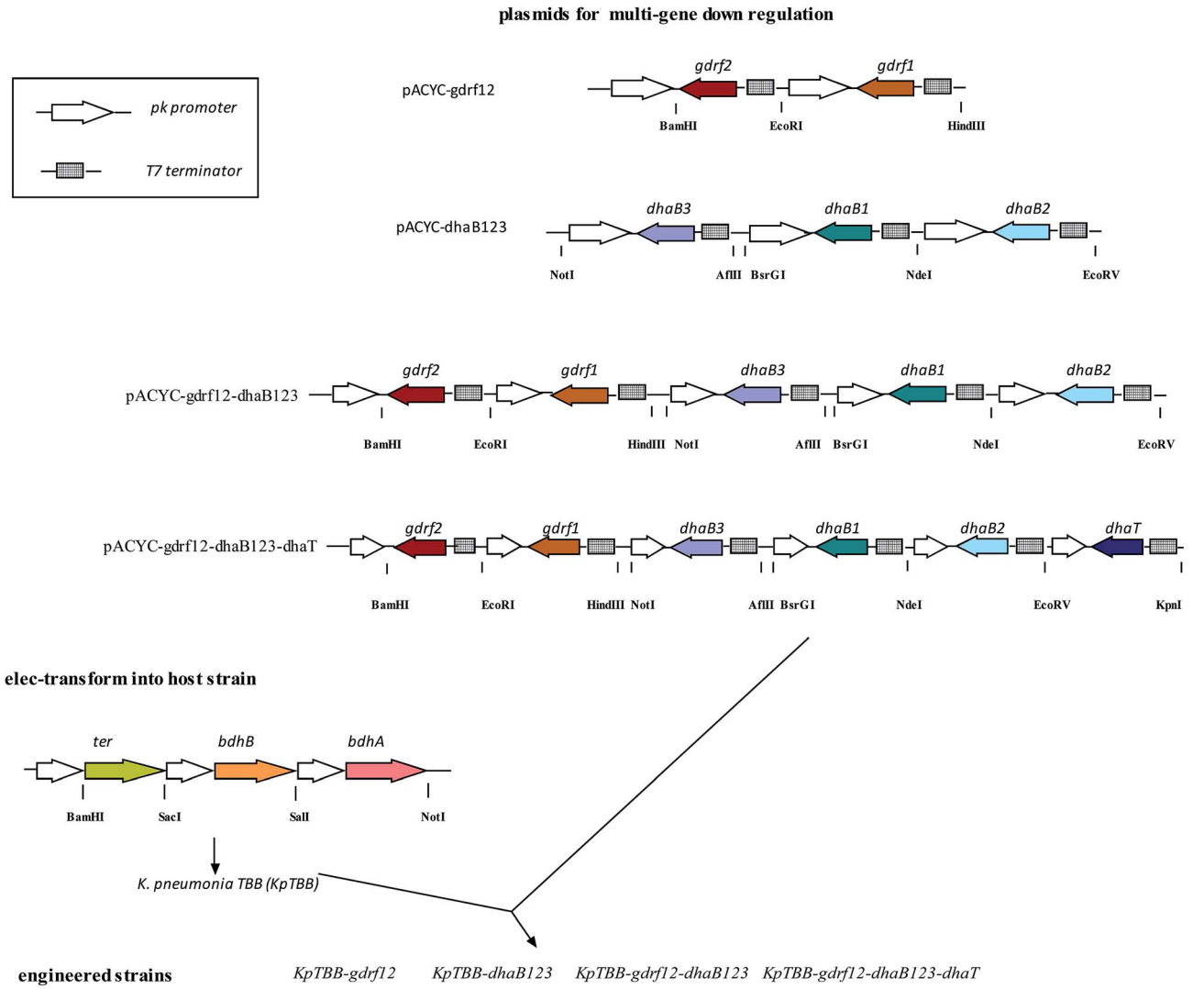

Fig. 1 Strategy for plasmids and strain construction. Oppositely inserted a 300-nucleotide molecule with 100% complementarity in a 300-nucleotide region of the mRNA of the specific gene was adopted to down regulate the special gene. The plasmids used for single gene down regulation were constructed by oppositely inserting a single gene with *Bam*H I and *Avr* II. The gene *gdrf1* encoded glycerol dehydratase-reactivation factor 1, the gene *gdrf2* encoded glycerol dehydratase-reactivation factor 2, the genes *dhaB123* encoded glycerol dehydratase, the gene *dhaT* encoded 1,3-propanediol oxidoreductase, the gene *alaS* encoded acetolactate synthase, and the gene *budC* encoded acetoin reductase.

medium[17] (5 g glucose, 20 g glycerol, 3 g yeast extract, 1.3 g KH_2PO_4, 3.4 g $K_2HPO_4 \cdot 3H_2O$, 0.24 g $MgSO_4 \cdot 7H_2O$, 3.0 g $(NH_4)_2SO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$ per liter water) and 1000 × trace element solution (2.72 g $ZnCl_2 \cdot 6H_2O$, 32 g $FeSO_4$, 0.68 g $MnCl_2 \cdot 4H_2O$, 1.88 g $CoCl_2 \cdot 6H_2O$, 0.24 g H_3BO_3, 0.02 g Na_2MoO_4, 1.88 g $CuCl_2 \cdot 2H_2O$, and 40 mL conc. HCl per liter water).

The medium mentioned above was sterilized by autoclaving at 116 °C for 25 min. Before inoculation, Kanamycin and/or Chloromycetin were added for selection of the recombinant strains, and the final concentrations were 50 mg L^{-1} and 85 mg L^{-1}, respectively.

Micro-aerobic conditions were achieved using a 100 mL Erlenmeyer flask containing 50 mL medium followed by shaking at 140 rpm for the first 4 h, and then at 70 rpm until the end. The temperature and growth period were fixed at 37 °C and 24 h, respectively. The flask fermentation for decreasing by-products was carried out at 37 °C and 140 rpm for the whole culture period.

2.4. Assays

The cell density was analyzed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (Thermo Scientific, USA). The specific growth rates were estimated from the OD_{600} data.

1-Butanol was analyzed by gas chromatography (GC) (GC-2010 Shimazu, Japan) equipped with a FID detector and DB-FFAP capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness) from Agilent Technologies.[16] For the other metabolites, glycerol, 1,3-PDO, and 2,3-BD, filter supernatant was

Table 2 Primer sequences

Primer names	Sequence 5′ → 3′
kivd-F (*Bam*H I)	CAC*GGATCC*ATGTACACTGTCGGTGACTACCT
kivd-R (*Sac* I)	GAC*GAGCTC*TTAGGACTTGTTCTGTTCAGCGAAC
ter-F (*Bam*H I)	CAC*GGATCC*ATGATCGTTAAACCGATGG
ter-R (*Sac* I)	ACA*GAGCTC*TTAGATACGGTCG
bdhB-F (*Bam*H I)	CG*GGATCC*GTGGTTGATTTCGAATATT
bdhB-R (*Sac* I)	CGC*GAGCTC*TTACACAGATTTTTTG
bdhA-F (*Bam*H I)	CG*GGATCC*ATGCTAAGTTTTGATTATTC
bdhA-R (*Sac* I)	CGC*GAGCTC*TTAATAAGATTTTTT
pk-bdhB-F (*Sac* I)	ATA*GAGCTC*CGTTATTTTGTCGCC
pk-bdhB-R (*Sal* I)	CGG*GTCGAC*TTACACAGATTTTTTG
pk-bdhA-F (*Sal* I)	ATA*GTCGAC*CGTTATTTTGTCGCCCGCCAT
pk-bdhA-R (*Not* I)	CGC*GCGGCCGC*TTAATAAGATTTTTTAAATATC
as-dhaB3-F (*Avr* II)	CGC*CCTAGG*CGGCAAACCATTGACCGATATT
as-dhaB3-R (*Bam*H I)	ATA*GGATCC*CGGACAAAGGCGGCATTCAC
as-dhaB2-F (*Avr* II)	ACA*CCTAGG*GGGGATCGGCATCGGTATCC
as-dhaB2-R (*Bam*H I)	ACA*GGATCC*CTTACTAAGTCGACGTGCAGGGTG
as-dhaB1-F (*Avr* II)	CGC*CCTAGG*TTGACATGATCGACCGGTTTATC
as-dhaB1-R (*Bam*H I)	ATA*GGATCC*CGGCGTCAGCGGCAATCTG
as-gdrf1-R (*Avr* II)	ATA*CCTAGG*CTTCTTCGGGCTAAGCCCGGAAGAG
as-gdrf1-F (*Bam*H I)	CAC*GGATCC*CGCCAGCAGATCCTGGATGTATATC
as-gdrf2-R (*Avr* II)	ACA*CCTAGG*GCCAGGCGTACGCCTGTTTTACGAT
as-gdrf2-F (*Bam*H I)	CGC*GGATCC*AGTTTCTCTCACTTAACGGCAGGAC
as-dhaT-R (*Avr* II)	CAC*CCTAGG*TCCGCTGCTGATGATCGGTAAACCG
as-dhaT-F (*Bam*H I)	ACA*GGATCC*ATCAGGTTGTAGCGCGCCACATGCG
as-alaS-F (*Avr* II)	CGC*CCTAGG*CCATATCTGGATTGCCCGCTACC
as-alaS-R (*Bam*H I)	ACC*GGATCC*CCAAACTCGACGCCGGACAG
as-budC-F (*Avr* II)	CGC*CCTAGG*GAGGGTCACGGCGGGAAAAT
as-budC-R (*Bam*H I)	ATC*GGATCC*GACATCTTCCGGCTCGGACAGG
pk-gdrf2-R (*Eco*R I)	CGC*GAATTC*CAAAAAACCCCTCAAGACCCGTT
pk-gdrf1-F (*Eco*R I)	ACG*GAATTC*CGTTATTTTGTCGCCCGCCATGATT
pk-gdrf1-R (*Hin*d III)	CGC*AAGCTT*CAAAAAACCCCTCAAGACCC
pk-dhaB1-F (*Bsr*G I)	ACG*TGTACA*CGTTATTTTGTCGCCCGCCATGATT
pk-dhaB1-R (*Nde* I)	CGG*CATATG*CAAAAAACCCCTCAAGACCC
pk-dhaB2-F (*Nde* I)	ACG*CATATG*CGTTATTTTGTCGCCCGCCATGATT
pk-dhaB2-R (*Eco*R V)	CGC*GATATC*CAAAAAACCCCTCAAGACCC
pk-dhaB3-F (*Not* I)	ATA*GCGGCCGC*CGTTATTTTGTCGCCCGCCATGATT
pk-dhaB3-R (*Afl* II)	CGCCGC*CTTAAG*CAAAAAACCCCTCAAGACCC
pk-dhaT-F (*Eco*R V)	ACG*GATATC*CGTTATTTTGTCGCCCGCCATGATT
pk-dhaT-R (*Kpn* I)	CAT*GGTACC*CAAAAAACCCCTCAAGACCC
16sRNA-F	CCTACGGGAGGCAGCAG
16sRNA-R	ATTACCGCGGCTGCTGG
budC-F	CATCACGGTCAACGGCTACT
budC-R	TGGCAAGATAGGAGACGCAG
alaS-F	GGTGAATCAGGATAACTTCTCTCG
alaS-R	ATCACCAGGTCGGCAAGC
dhaB2-F	TGCCGATGAAGTGGTGATC
dhaB2-R	ATAAAGGAGACGTCGGACGT
dhaB3-F	GAGACCAAACATGTGGTGCA
dhaB3-R	AGGATATGCTCCGGGCAG
dhaT-F	ATCTGGTGCCAAACGTTAACT
dhaT-R	ACGATGATGTCGCACTGTTC
gdrf2-F	CTACATGGCGGAGATGGCT
gdrf2-R	GCGGTGAAAGCGACATGAC

applied (20 μL) to UltiMate 3000 HPLC (Thermo Scientific, USA) equipped with a Bio-Rad (Biorad Laboratories, USA) Aminex HPX-87H column (0.5 mM H_2SO_4, 0.6 mL min^{-1}, column temperature at 65 °C) with a RID detector.

The mRNA levels were measured and quantified by quantitative PCR (ABI, USA), which was performed by Sangon Biotech Co. Ltd. (Shanghai, China). The kits used for total RNA extraction, first strand cDNA synthesis, and real time analysis were obtained from Sangon (Shanghai, China). The reported RNA concentrations in Fig. 6 are expressed as a percentage of the 16S rRNA concentration within each sample.

3. Results and discussion

3.1. Establish 1-butanol synthesis pathway in *K. pneumoniae*

There are two distinct pathways for 1-butanol synthesis according to the literature [1–5, 8, 9, 13, 14], *i.e.*, CoA-dependent pathway naturally exists in *C. acetobutylicum* and 2-keto acid pathway that utilizes intermediates in the amino acid pathway. According to the database (KEGG, http://www.genome.jp/kegg/), several enzymes in *K. pneumoniae* have similar catalytic ability compared to those required in 1-butanol synthesis in *C. acetobutylicum*. Only the absent genes, *bcd*/*etfAB*, *bdhB* and *bdhA*, are to be introduced. To establish the CoA-dependent pathway in the host, the gene *ter* (Genebank no. TDE0597) was introduced with *bdhB* (Genebank no. CA_C3298) and *bdhA* (Genebank no. CA_C3299) to engineer the recombinant *KpTBB* (see Fig. 2), replacing the gene *bcd*/*etfAB* due to the sensitivity of Bcd/EtfAB complex to oxygen.[26]

In the 2-keto acid pathway, 1-butanol is produced from 2-ketovalerate, a rare metabolite, which is the intermediate in the synthesis of the unnatural amino acid-norvaline.[3] Several studies[3,5,27] have reported that LeuABCD which has broad substrate specificity has been overexpressed for 2-ketovalerate syntheses. On the other hand, Soini *et al.*[28] suggested that norvaline can be accumulated immediately after a shift to oxygen limitation. The oxygen limitation could give an impetus to the formation of 2-ketovalerate. Based on this, the engineered strain, *Kp-kivd*, was constructed by the introduction of only one gene, *kivd* (Genebank no. ADA65057.1) (see Fig. 2).

The wild-type strain, *KpTBB* and *Kp-kivd* were selected and tested for 1-butanol production in flask fermentation with glycerol as the substrate, and the results are shown in Fig. 3. It was easy to conclude that they had similar growth rates, the maximum peak of OD_{600} in the recombinant strains was slightly lower than the control strain, and the wild-type strain did not produce any detectable 1-butanol. 1,3-PDO and 2,3-BD were two major products in these fermentations. In contrast, significant amounts of 1-butanol were produced in both *KpTBB* and *Kp-kivd*, confirming that the introduction of *ter-bdhBA* and *kivd* into *K. pneumoniae* enabled the host cells to produce 1-butanol. The resulting 1-butanol titer and specific BuOH yield were 15 mg L^{-1} and 27.8 mg-BuOH per g cell in *KpTBB*, and 28.7 mg L^{-1} and 51.6 mg-BuOH per g cell in *Kp-kivd*, respectively.

The 1-butanol produced by the CoA-dependent pathway is lower than that produced by the 2-keto acid pathway. In Fig. 2, 1-butanol synthesis requires NADH as a cofactor, and the total NADH consumed by introduced reactions in the CoA-dependent pathway (from acetyl-CoA to 1-butanol) is not balanced. Although the reaction catalyzed by the gene *kivd* consumes one NADH molecule, another NADH molecule is produced during the synthesis of 2-ketovalerate by oxygen limitation. Therefore, the total consumption of NADH in the 2-keto acid pathway of 1-butanol production is balanced. Moreover, the 2-keto acid pathway circumvents the need for some toxicity metabolites in the host's protein synthesis and growth, especially CoA-dependent intermediates.[10] Utilizing the organism's native metabolites in the amino-acid biosynthetic pathway to produce 1-butanol is desirable.

On the other hand, the final 1-butanol titers obtained in these fermentations were low (15 mg L^{-1} in *KpTBB* and 28 mg L^{-1} in *Kp-kivd*), which can be attributed to the competition pathways that utilize glycerol for 1,3-PDO and 2,3-BD synthesis (shown in Fig. 2).

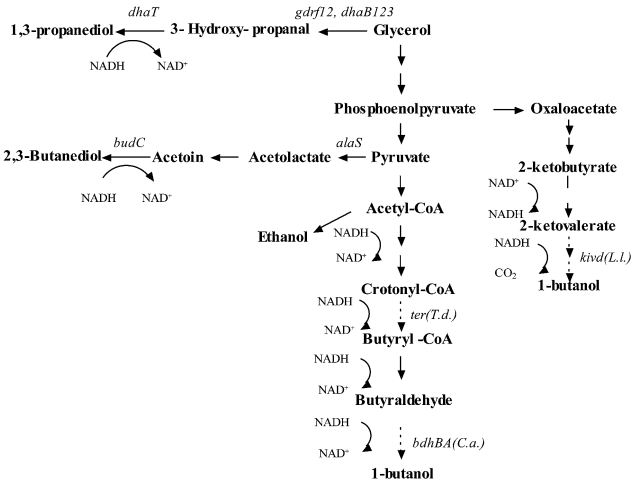

Fig. 2 Metabolic pathway in *K. pneumoniae*. The pathways for 1-butanol formation shown in the dotted lines are absent in wild-type *K. pneumoniae*. The introduced genes *bdhA* and *bdhB* were amplified from the *C. acetobutylicum* genome, and the gene *ter* and *kivd* amplified from *Treponema denticola* and *Lactococcus lactis* separately were introduced after codon optimization. The reaction shown in dash line was achieved by oxygen limited operation.

3.2. Down regulation of the by-products yield by the asRNA strategy

3.2.1 Down regulation of 1,3-PDO by asRNA strategy. In *K. pneumoniae*, the digestion of glycerol[17,24] is a dismutation process: glycerol is catalyzed to synthesize 1,3-PDO *via* the reductive pathway and 2,3-BD *via* the oxidative pathway. In the reductive pathway, glycerol is catalyzed by the glycerol dehydratase-reactivation factor (encoded by the genes *gdrf1* and *gdrf2*), glycerol dehydratase (encoded by the genes *dhaB123*) and 1,3-propanediol oxidoreductase (encoded by the gene *dhaT*) to synthesize 1,3-PDO (see Fig. 2). In the engineered *KpTBB* and *Kp-kivd*, 1-butanol is synthesized in the oxidative pathway. Therefore, the reductive pathway of glycerol is the key competitor to the production of 1-butanol in the engineered *K. pneumoniae*. In this section, the key enzymes included in 1,3-PDO synthesis were inactive by introducing asRNA, which could inhibit the normal expression of the target mRNA.[18,29]

The mRNA relative level of the genes mentioned above in engineered strains is shown in Fig. 4. The mRNA level of the genes *dhaB2*, *dhaB3*, *dhaT*, and *gdrf2* in the engineered strains was normalized by 16sRNA, and the normalized mRNA level of these genes in *KpTBB* was assumed to be 1.0. Compared to the control strain, the mRNA relative level of all the genes was reduced in all the engineered strains with the asRNA fragment. The peak levels of *dhaB2*, *dhaB3*, *dhaT*, and *gdrf2* in all the

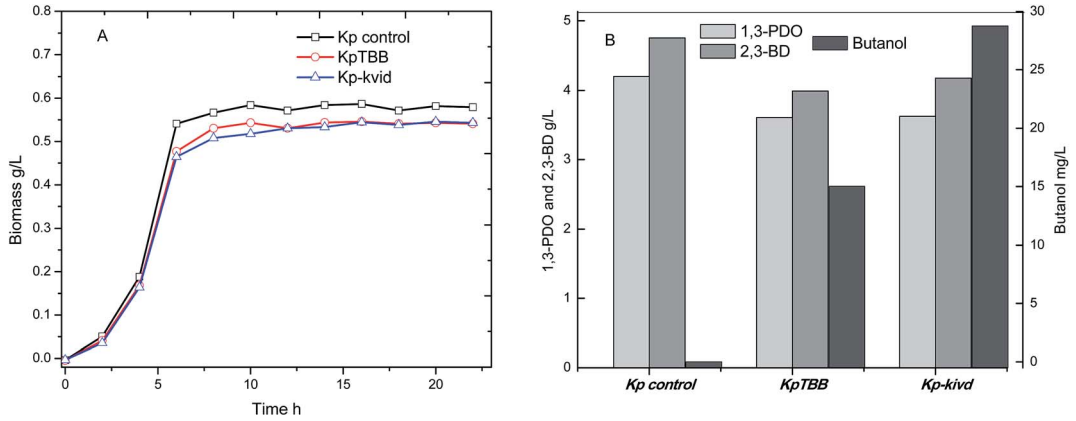

Fig. 3 Flask fermentation result of *Kp* control, *KpTBB* and *Kp-kivd*.

engineered strains were 55–61%, 51–68%, 42–53%, and 19–65% lower, respectively, than the control levels.

The impact of the produced asRNA of the relative genes, included in 1,3-PDO synthesis, on the growth and product formation is summarized in Fig. 5. Although the engineered and control strains had similar growth rates (data not shown) and reached a similar peak OD_{600}, the yield of 1,3-PDO changed significantly. In Fig. 5A, it decreased by 56–62% when the genes *dhaB1*, *dhaB2*, *dhaB3*, *gdrf1*, and *gdrf2* were silenced separately. The most obvious down regulated effect was achieved by introducing *dhaT*-asRNA, and the resulting production of 1,3-PDO was reduced by 71%. In Fig. 5B, the 1,3-PDO yield decreased by 67–82% when more than one gene were silenced. The 1,3-PDO yield of *KpTBB-gdrf12-dhaB123-dhaT* decreased by 82% compared to that of the wild type strain, which was the lowest. The production of 1-butanol in all of these engineered *K. pneumoniae* was increased in varying degrees compared to that of *KpTBB*. The maximum yield of 1-butanol was obtained in *KpTBB-gdrf2* (increased by 41%) among those engineered strains, all of which were constructed to down regulate the relative single gene included in 1,3-PDO syntheses. When the genes *gdrf12* and *dhaB123* were silenced together, the resulting 1-butanol production was increased by 37%, which was higher than that of the engineered strains in Fig. 5B.

The 1,3-PDO titer has been effectively decreased by adopting the asRNA strategy, and the final 1-butanol titer increased to varying degrees in the low 1,3-PDO producing strain.

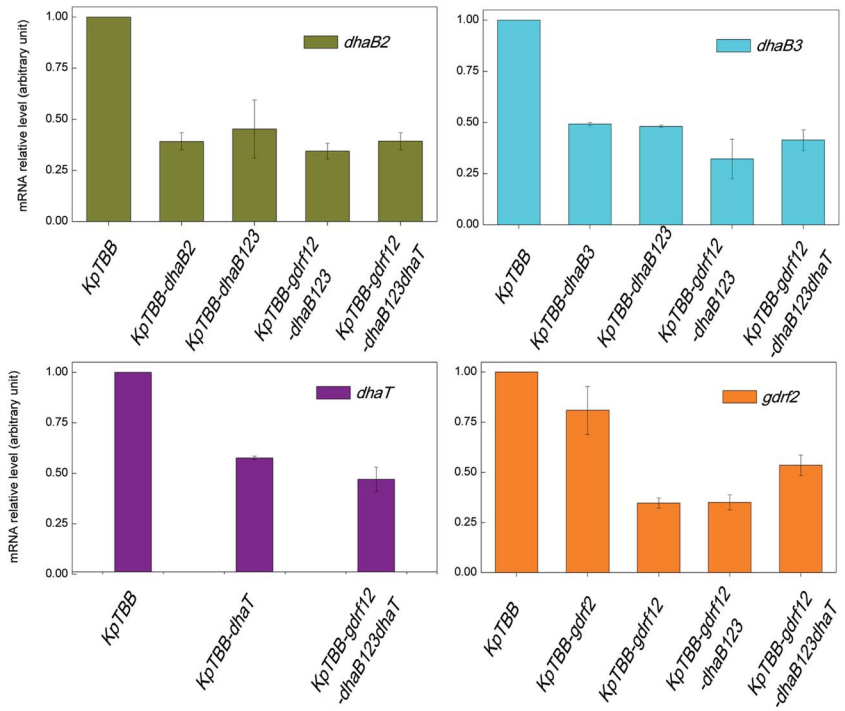

Fig. 4 mRNA level of the genes included in 1,3-PDO synthesis pathway in the engineered strains.

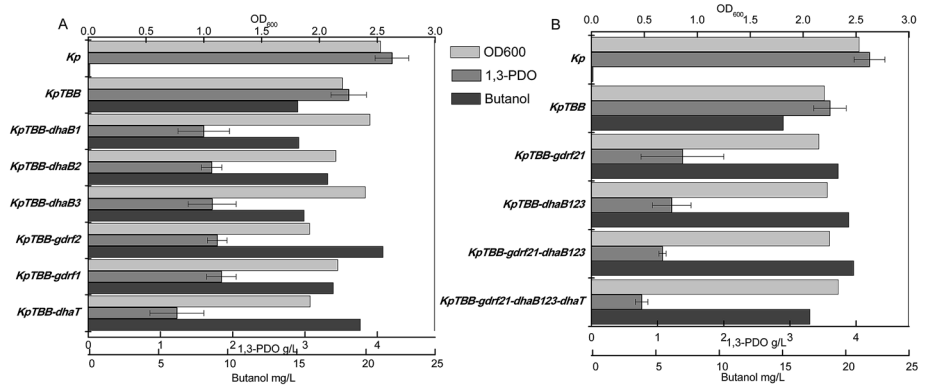

Fig. 5 Flask fermentation results of the *Kp* control strain and engineered strains. (A) Downregulates the single gene and (B) down regulate the multi genes.

3.2.2 Downregulation of 2,3-BD yield by asRNA strategy. 2,3-BD is another important product in the fermentation of control *K. pneumoniae*. In this section, the downregulative effect of the 2,3-BD yield with asRNA strategy was investigated. The impact of *alaS*-asRNA/*budC*-asRNA on the mRNA level was studied, and 16sRNA was used as an internal reference. The mRNA relative level of *alaS* and *budC* in engineered strains is shown in Fig. 6.

The mRNA level of *alaS* and *budC* in the engineered strains was normalized by 16sRNA, and the normalized mRNA level of *alaS* and *budC* in the *K. pneumoniae* parent was assumed to be 1.0. As shown in Fig. 6, the mRNA relative level of *budC* in *KpTBB-budC* was almost 92% lower than that in the control strain, and the mRNA level of *alaS* in *KpTBB-alaS* was 16% lower than that in the control strain.

The impact of the introduced *alaS*-asRNA/*budC*-asRNA on cell growth and product formation is summarized in Fig. 7. The engineered and control strains had similar growth rates (data not shown), but the peak OD_{600} in the engineered strains was much lower than that in the control strain. Both cell growth and the production of 2,3-BD were influenced by *alaS*-asRNA/*budC*-asRNA. The production of 2,3-BD in the control strain was 8.4 g L^{-1}. When adopting the asRNA strategy, the level of 2,3-BD production of *KpTBB-alaS* and *KpTBB-budC* was reduced by 13% and 15%, respectively, compared to that of the control strain.

Both the transcriptional level of *alaS* and *budC* and the 2,3-BD titer were decreased significantly by the asRNA strategy. The larger decrease in the mRNA relative level, however, did not come with a larger decrease in the 2,3-BD titer. To reduce the mRNA relative level, the introduced the antisense fragment of the gene *budC* was more effective than that of the gene *alaS*. On the other hand, the effect for decreasing the 2,3-BD was similar regardless of the introduction of the antisense fragment of *alaS* or *budC*. This may be due to the different roles of the genes and enzymes involved in one pathway.

The asRNA strategy is a very useful and effective method for silencing the normal expression of some specific genes, and it can down regulate the concentration of related metabolites, particularly for those strains lacking a suitable gene knockout tool. The asRNA strategy has been deduced to disturb the normal translation process by hybridizing with complementary mRNA. As for how it works several mechanisms have been

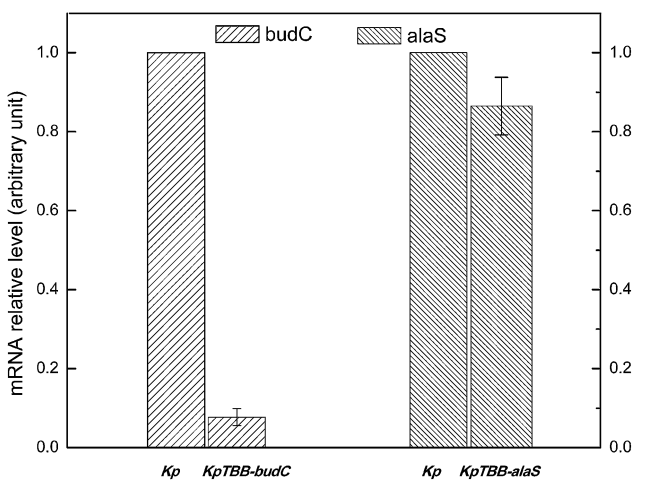

Fig. 6 mRNA level of *alaS* and *budC* gene in the engineered strains.

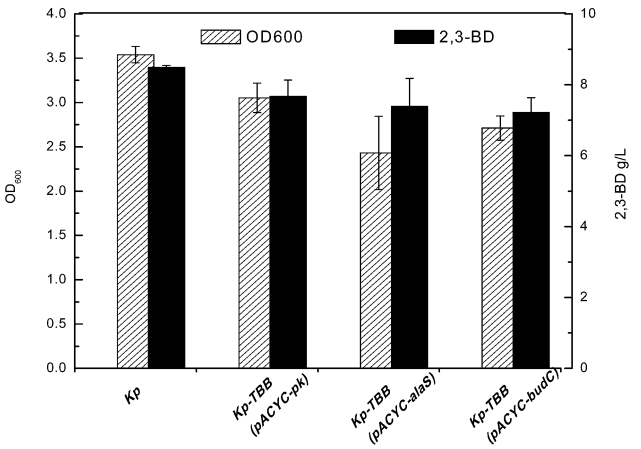

Fig. 7 Production of 2,3-BD and OD_{600} in the *Kp* control strain and engineered strains. Four independent experiments were carried out, and the average results and standard deviation are shown in this figure.

postulated,[18] such as the prevention of access to the ribosome binding sites by the duplex RNA structure, the degradation of the mRNA catalyzed by duplex RNA-specific RNases or the inhibition of transcription of mRNA due to premature termination. Based on the 2,3-BD titer and the mRNA level of specific genes in the engineered strains, downregulation of the metabolite by the asRNA strategy may be due to the degradation of the mRNA. RNA–RNA duplex-specific RNases that can decay the duplex RNA rapidly might also exist. Using the asRNA strategy, a splice of reverse complemented DNA was introduced to the host. This produced a splice of mRNA with a reverse complementary sequence of the specific mRNA transcribed by the genome. The duplex RNA formed after these two mRNA hybridized with each other, and then decayed by RNA–RNA duplex-specific RNases. The decrease in mRNA content resulted in the effective decrease in by-products yield.

4. Conclusion

Here, we engineered *K. pneumoniae* to produce 1-butanol using glycerol, the by-product from the production of biodiesel. Two distinct butanol production pathways were successfully constructed. Regarding the two introduced 1-butanol production pathways, the 2-keto acid pathway is more effective. The antisense RNA strategy can significantly affect primary metabolite production. Using this strategy, 1,3-PDO and 2,3-BD, were reduced by 81% and 15%, respectively. A comparison of our strain with other 1-butanol producers, (*Clostridia* species) ($\sim$10 g L^{-1})[13] and engineered *E. coli* strains ($\sim$1 g L^{-1}),[26] provides a goal for future 1-butanol titers. Given the results mentioned above and the other advantages of *K. pneumoniae*, such as growing on glycerol as the sole carbon source, having a similar genetic background to *E. coli* and exhibiting rapid cell proliferation (even faster than *E. coli*), *K. pneumoniae* may be an very promising host to be engineered to produce 1-butanol. Studies aimed at increasing the product titer are currently underway.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 program) (2013CB733600, 2012CB725200), the National Nature Science Foundation of China (21390202, 21436002), National Key Scientific Instruments and Equipment Development Special Fund (2012YQ0401400302).

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