Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-5526-9

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Mechanism of 2,3-butanediol stereoisomer formation in *Klebsiella pneumoniae*

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Received: 16 October 2013 / Revised: 7 January 2014 / Accepted: 7 January 2014

Abstract *Klebsiella pneumoniae* is known to produce *meso*-2,3-butanediol and 2*S*,3*S*-butanediol, whereas 2*R*,3*R*-butanediol was detected in the culture broth of *K. pneumoniae* CGMCC 1.6366. The ratio of 2*R*,3*R*-butanediol to all isomers obtained using glycerol as the carbon source was higher than that obtained using glucose as the carbon source. Therefore, enzymes involved in glycerol metabolism are likely related to 2*R*,3*R*-butanediol formation. In vitro reactions show that glycerol dehydrogenase catalyzes the stereospecific conversion of *R*-acetoin to 2*R*,3*R*-butanediol and *S*-acetoin to *meso*-2,3-butanediol. Butanediol dehydrogenase exhibits high (*S*)-enantioselectivity in ketone reduction. Genes encoding glycerol dehydrogenase, α-acetolactate decarboxylase, and butanediol dehydrogenase were individually disrupted in *K. pneumoniae* CGMCC 1.6366, and the 2,3-butanediol synthesis characteristics of these mutants were investigated. *K. pneumoniae* ΔdhaD lost the ability to synthesize 2*R*,3*R*-butanediol. *K. pneumoniae* ΔbudA showed reduced 2*R*,3*R*-butanediol synthesis. However, *K. pneumoniae* ΔbudC produced a high level of 2*R*,3*R*-butanediol, and *R*-acetoin was accumulated in the broth. The metabolic characteristics of these mutants and in vitro experiment results demonstrated the mechanism of the 2,3-butanediol stereoisomer synthesis pathway. Glycerol dehydrogenase, encoded by *dhaD*, exhibited 2*R*,3*R*-butanediol dehydrogenase activity and was responsible for 2*R*,3*R*-butanediol synthesis from *R*-acetoin. This enzyme also contributed to *meso*-2,3-butanediol synthesis from *S*-acetoin. Butanediol dehydrogenase, encoded by *budC*, was the only enzyme that catalyzed the conversion of diacetyl to *S*-acetoin and further to 2*S*,3*S*-butanediol.

Keywords 2,3-Butanediol · Stereoisomer · *Klebsiella pneumoniae* · α-Acetolactate decarboxylase · Butanediol dehydrogenase · Glycerol dehydrogenase

Introduction

2,3-Butanediol is a bulk chemical that can be produced by biotechnological routes. 2,3-Butanediol and its derivatives have applications in the field of plastics, solvent production, and synthetic rubber and have the potential to be used as biofuel (Celinska and Grajek 2009). Numerous microorganisms can produce 2,3-butanediol, among which *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, and *Bacillus polymyxa* are efficient producers that have the potential to be used in industrial applications.

The metabolic pathway of 2,3-butanediol synthesis in *K. pneumoniae* (*Aerobacter aerogenes*) was identified by Stormer (1975). The genes involved in 2,3-butanediol synthesis have been identified in *Klebsiella terrigena* (Blomqvist et al. 1993). 2,3-Butanediol is synthesized from pyruvate whereby two molecules of pyruvate condense to yield α-acetolactate and release one molecule of CO_2. This reaction is catalyzed by α-acetolactate synthase. α-Acetolactate is then converted to acetoin by α-acetolactate decarboxylase. Finally, acetoin is reduced to 2,3-butanediol by butanediol dehydrogenase (see dashed rectangle in Fig. 1). α-Acetolactate produced in the process is unstable and can be converted to diacetyl as a minor by-product by nonenzymatic oxidative

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Published online: 18 February 2014

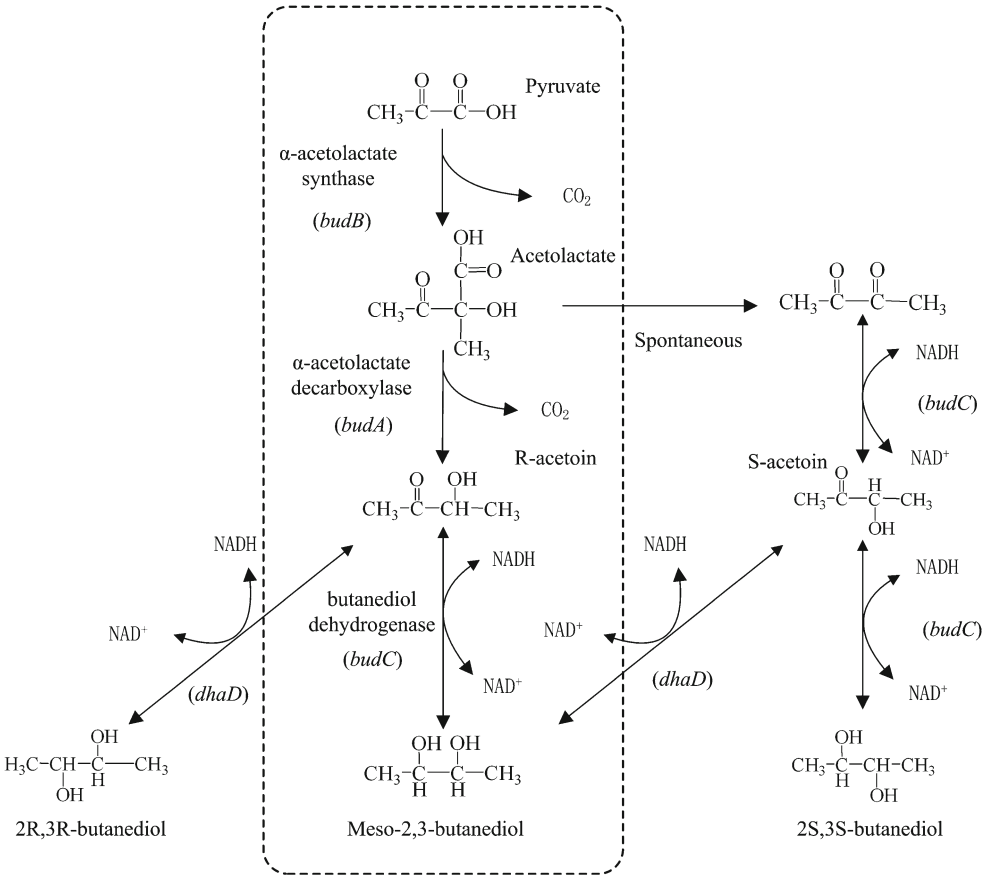


Fig. 1 2,3-Butanediol synthesis pathway and mechanism of 2,3-butanediol stereoisomer formation. *budC* encodes butanediol dehydrogenase, and *dhaD* encodes glycerol dehydrogenase. *Dashed rectangle* indicates 2,3-butanediol synthesis pathway identified by Stormer (1975)

decarboxylation (Xiao and Xu 2007). The three enzymes in the butanediol synthesis pathway are encoded by *budB*, *budA*, and *budC*. The three structural genes and a regulatory gene *budR* form the *bud* operon.

The molecule of 2,3-butanediol contains two chiral carbons, resulting in three stereoisomers, namely, 2*R*,3*R*-butanediol (D-butanediol), 2*S*,3*S*-butanediol (L-butanediol), and *meso*-2,3-butanediol. Reports have shown that *Klebsiella* spp. produces *meso*-2,3-butanediol together with a small amount of 2*S*,3*S*-butanediol, whereas *Bacillus* spp. produces 2*R*,3*R*-butanediol accompanied by *meso*-2,3-butanediol (Ui et al. 1986). Voloch et al. (1983) have proposed a model for the formation of 2,3-butanediol stereoisomers in *K. pneumoniae*. The model postulates the presence of an acetoin racemase and two acetoin reductases. One acetoin reductase catalyzes the reduction of *R*-acetoin (D-acetoin) to *meso*-2,3-butanediol but does not use *S*-acetoin (L-acetoin). Another acetoin reductase catalyzes the reduction of *S*-acetoin to 2*S*,3*S*-butanediol but does not reduce *R*-acetoin. Ui et al. (1984) have proposed another model for the formation of stereoisomers in *K. pneumoniae* which includes the presence of three butanediol dehydrogenases: *meso*-butanediol dehydrogenase (*R*-acetoin forming), *meso*-butanediol dehydrogenase (*S*-acetoin forming), and 2*S*,3*S*-butanediol dehydrogenase. However, they did not confirm the presence of acetoin or butanediol racemase.

The aforementioned two models have been proposed prior to the full development of molecular biology. Thus, evidences were obtained from the enzyme reactions that used cellular extracted fractions. A dozen years later, the gene encoding *meso*-butanediol dehydrogenase (*R*-acetoin forming) has been cloned and expressed in *Escherichia coli*. This enzyme catalyzes the stereospecific conversion of *R*-acetoin to *meso*-2,3-butanediol. When diacetyl is used as a substrate, diacetyl is converted to *S*-acetoin and then further converted to 2*S*,3*S*-butanediol (Ui et al. 1997, 1999). The model proposed by Ui et al. shows the existence of three butanediol dehydrogenases, but only the *meso*-butanediol dehydrogenase (*R*-acetoin forming) gene has been cloned in *K. pneumoniae*. The sequence indicates that this gene is *budC*, which is applied for *meso*-2,3-butanediol production using *R*-acetoin as a substrate (Yan et al. 2009).

The strain *K. pneumoniae* CGMCC 1.6366 (TUAC01) was isolated for 1,3-propanediol production (Hao et al. 2008). When glycerol was used as the carbon source, the main metabolic product of this strain was 1,3-propanediol, with some 2,3-butanediol as by-product. However, the main metabolic product was 2,3-butanediol when glucose was used as the carbon source. The stereoisomers of the 2,3-butanediol produced by this strain were analyzed, and it was found to contain all three stereoisomers. 2*R*,3*R*-Butanediol produced

by *K. pneumoniae* has not been reported elsewhere. Therefore, the mechanism of 2,3-butanediol stereoisomer formation in *K. pneumoniae* is investigated here. The ratio of 2*R*,3*R*-butanediol to all isomers obtained using glycerol as the carbon source was higher than that obtained using glucose as the carbon source. Therefore, enzymes involved in glycerol metabolism are likely related to 2*R*,3*R*-butanediol formation. Glycerol dehydrogenase was found to exhibit 2*R*,3*R*-butanediol dehydrogenase activity. The complete metabolic pathway of 2,3-butanediol stereoisomer formation is presented in this study.

Materials and methods

Strains, plasmids, and primers

Bacteria strains and plasmids are listed in Table 1. Primers used for PCR are listed in Table 2.

Medium and culture condition

K. pneumoniae and *E. coli* were cultured in Luria–Bertani (LB) medium at 37 °C. When necessary, the medium was supplemented with ampicillin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}), or streptomycin (25 μg mL^{-1}).

The fermentation medium contained glucose 50 g/L or glycerol 30 g/L, corn steep liquor 4 g/L, $(NH_4)_2SO_4$ 5 g/L, sodium acetate 3 g/L, KCl 0.4 g/L, and $MgSO_4$ 0.1 g/L. For the seed culture, 250-mL flasks containing 50 mL of LB medium were incubated in a rotary shaker at 37 °C and 200 rpm overnight. A total 50 mL of seed culture was inoculated into a 5-L bioreactor (BIOSTAT A plus Sartorius) with a working volume of 3 L. The air supplement, agitation, and culture temperature were 4 L/min, 250 rpm, and 37 °C, respectively. The culture pH was automatically controlled to be 6 by the addition of 10 M NaOH. Glucose or glycerol solution was fed to the bioreactor when the glucose level in the medium decreased to 20 g/L or the glycerol level decreased to 10 g/L.

Construction of *K. pneumoniae* ΔbudC and *K. pneumoniae* ΔdhaD

The *budC* mutant strain of *K. pneumoniae* was constructed following a previously described method (Wei et al. 2012). Briefly, the *budC* gene in *K. pneumoniae* and flanking sequences were amplified by PCR using the primer pair budC-s1/budC-a1. The PCR product was ligated into the pMD18-T

Table 1 Strains and plasmids

Strain or plasmid	Relevant genotype and description	Reference or source
Strains		
E. coli DH5α	Host of plasmid	Lab stock
E. coli BL21	Host of plasmid	Lab stock
E. coli BL21/budC	*E. coli* BL21/budC carries pET28a-budC	This work
E. coli BL21/dhaD	*E. coli* BL21/dhaD carries pET28a-dhaD	This work
K. pneumoniae CGMCC 1.6366	Wild type	Hao et al. (2008)
K. pneumoniae/red	*K. pneumoniae* CGMCC 1.6366, pDK6-red	Wei et al. (2012)
K. pneumoniae ΔbudA	*K. pneumoniae* CGMCC 1.6366, ΔbudA	Wei et al. (2013)
K. pneumoniae ΔbudC	*K. pneumoniae* CGMCC 1.6366, ΔbudC	This work
K. pneumoniae ΔdhaD	*K. pneumoniae* CGMCC 1.6366, ΔdhaD	This work
Plasmids		
pDK6-red	Kan^r, carries λ-Red genes (gam, bet, exo), 7.1 kb	Wei et al. (2012)
pET28a	vector carries an N-terminal His Tag, Kan^r, 5,369 bp	Novagen®
pET28a-budC	pET28a carries *budC*, 6,140 bp	This work
pET28a-dhaD	pET28a carries *dhaD*, 6,464 bp	This work
pMD18-T simple	Amp^r, TA cloning vector, 2,692 bp	Takara
pMD18-T-budC	Amp^r, carries *budC* with, 4,109 bp	This work
pMD18-T-budC2	Amp^r, carries *budC2* with, 3,649 bp	This work
pMD18-T-ΔbudC	Amp^r, carries *ΔbudC* with, 5,111 bp	This work
pMD18-T-dhaD	Amp^r, carries *dhaD* with, 4,359 bp	This work
pMD18-T-dhaD2	Amp^r, carries *dhaD2* with,3,787 bp	This work
pMD18-T-ΔdhaD	Amp^r, carries *ΔdhaD* with, 5,154 bp	This work
pIJ778	str^r, *aadA* with FRT sites, 4,377 bp	Gust et al. (2003)

Table 2 Oligonucleotides used for PCR

Primer name	Sequence (5′–3′)
budC-s	CGCCCGTCAGGTGATGATCTCCAAC
budC-s1	GCCATCCAGGAAGAGAAAAAATATCA
budC-a1	AGACGTTTGTACGCCTGGGTAGAAG
budC-s2	AGATAGGAGACGCAGGCGGCGACATCTTCCGGTT CGGACATTCCGGGGATCCGTCGAC
budC-a2	CAGGCGCGCAAAACGCTGGGCGGCTTCGACGTCA TCGTCATGTAGGCTGGAGCTGCTTC
budC-s3	CATATGAAAAAAGTCGCACTTGTTACCG
budC-a3	AAGCTTAGTTAAATACCATCCCGCCG
dhaD-s	TATGATGATTCTGGCTGAGCGGACG
dhaD-a1	GGTAAGGGAATTATGCGGCAGAGG
dhaD-s1	TCCATAAACTCCCAAGCGTCCTCC
dhaD-a2	AGAGTCACGATATTCGCTGCCATGCGGAACGGTT TAACGATTCCGGGGATCCGTCGACC
dhaD-s2	GGCCACTTTCTCGCCGTGATACAGGTGATGGCAC TCTTCCTGTAGGCTGGAGCTGCTTC
dhaD-a3	CATATGTTAACGCGCCAGCCACTGC
dhaD-s3	CATATGCTAAAAGTTATTCAATCTCCAG
Test778	AGAATCTCGCTCTCTCCAGGGGAAG

simple vector to generate pMD18-T-budC. A linear DNA with 39- and 40-nt homologous extensions flanking the streptomycin resistance gene *aadA* was amplified with plasmid pIJ778 as the template using the primer pair budC-s2/budC-a2. pMD18-T-ΔbudC was constructed by replacing the *budC* in plasmid pMD18-T-budC with the *aadA* cassette using the Red/ET system in *E. coli*. The pMD18-T-ΔbudC was further used as the template for PCR preparation of a linear DNA containing the streptomycin resistance gene *aadA* with 500-bp homologous regions at both sides. Finally, the linear DNA was transformed into *K. pneumoniae*/red, which already hosted the plasmid pDK6-red. Homologous recombination between the linear DNA and the chromosome was facilitated by Red recombinase and led to *budC* deletion in *K. pneumoniae* CGMCC 1.6366. The mutant was isolated on streptomycin plates, and the primer pair Test778 and budC-s were used for PCR confirmation.

The *dhaD* mutant strain of *K. pneumoniae* was constructed following the same method used for the *budC* mutant. The primers used were dhaD-s1/dhaD-a1, dhaD-s2/dhaD-a2, and Test778/dhaD-s.

Construction of *E. coli* BL21/budC and *E. coli* BL21/dhaD

The ORF of *budC* in *K. pneumoniae* CGMCC 1.6366 was amplified using the primer pair budC-s3 and budC-a3, which contain the *Nde* I and *Hin*d III restriction sites, respectively. The PCR product was ligated into the pMD18-T simple vector to generate pMD18-T-budC2. pMD18-T-budC2 was digested with *Nde* I and *Hin*d III to obtain the *budC* fragment, and this fragment was ligated into pET28a to generate pET28a-budC. pET28a-budC was transformed into *E. coli* BL21 for protein expression.

E. coli BL21/dhaD was constructed using the same approach as *E. coli* BL21/budC, using primers dhaD-s3 and dhaD-a3, and the *Nde* I site was used in dhaD-s3 and dhaD-a3.

Enzyme preparation, assay, and enzymatic reactions

E. coli BL21/budC and *E. coli* BL21/dhaD were incubated at 37 °C in the LB medium, and expression was induced at a 6-h culture with IPTG at a final concentration of 1 mmol/L. Cells were harvested 4 h after induction, and cell lysate was prepared by sonication. The cell lysate was centrifuged at 10,000*g* for 10 min to eliminate residual cells and debris. The enzyme in the supernatant was purified by affinity chromatography using a His-Trap column (5 ml) on an AKTA purifier (GE Healthcare).

The glycerol dehydrogenase activity assay was performed as described by Forage and Foster (1982), with minor modification. For oxidization reactions, the assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0) at 30 °C, 30 mM ammonium sulfate, 2.0 mM NAD, the enzyme fraction, and 0.1 M glycerol. The reaction was started by adding NAD, and the increase in absorbance at 340 nm was recorded with a NanoDrop 2000c spectrophotometer. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min.

For reduction reactions, NAD was replaced by NADH, and one unit of enzyme was defined as the amount of enzyme that catalyzes the consumption of 1 μmol of NADH per min.

The butanediol dehydrogenase activity assay was performed in the same way as the assay for glycerol dehydrogenase except that the appropriate substrate and enzyme fraction were used.

The enzymatic reactions were carried out similar to the assay method, except that the substrate concentration was high. Samples were collected during the process, and the chemicals were identified by gas chromatography.

Chemicals used in the enzyme assay and enzymatic reactions

Racemic acetoin and diacetyl were purchased from Sinopharm Chemical Reagent Co., Ltd. *R*-acetoin was purified from the fermentation broth of *K. pneumoniae* ΔbudC.

Analytical methods

Volatile chemicals in the broth were quantified using a gas chromatograph system (Shimadzu GC-2010) equipped with a flame ionization detector and a Rt®-bDEXse column (30 m×

0.25 mm), with nitrogen as the carrier gas. The injector and detector were both maintained at 225 °C. The column temperature initially started at 50 °C, increased to 75 °C at a rate of 5 °C/min, then maintained for 8 min, then increased to 200 °C at a rate of 20 °C/min, and maintained for 2 min. The culture samples were centrifuged to remove cells and debris, and the supernatants were mixed with the same amount of ethanol. Isopropanol was used as the internal standard.

Results

Metabolic characteristics of *K. pneumoniae* ΔbudA, *K. pneumoniae* ΔbudC, and *K. pneumoniae* ΔdhaD using glucose as the carbon source

K. pneumoniae ΔbudA, *K. pneumoniae* ΔbudC, and *K. pneumoniae* ΔdhaD were constructed as described in "Materials and methods." *budA* and *budC* encode α-acetolactate decarboxylase and butanediol dehydrogenase, which are the key enzymes in the 2,3-butanediol synthesis pathway. *dhaD* encodes glycerol dehydrogenase, which is in the *dha* regulon that is responsible for anaerobic glycerol metabolism and 1,3-propanediol production. Glycerol is its native substrate; however, this enzyme also catalyzes the dehydrogenate reaction of other diols. Thus, we hypothesized that glycerol dehydrogenase is responsible for 2,3-butanediol isomer formation. The three mutants and the wild-type strain were cultured in fermentation medium to investigate their metabolic characteristics. The cultures were conducted in a 5-L bioreactor, and the products in the broth were analyzed by GC. The results are shown in Fig. 2. The *meso*-butanediol dehydrogenase and glycerol dehydrogenase activities of the four strains were analyzed, and the results are shown in Fig. 3.

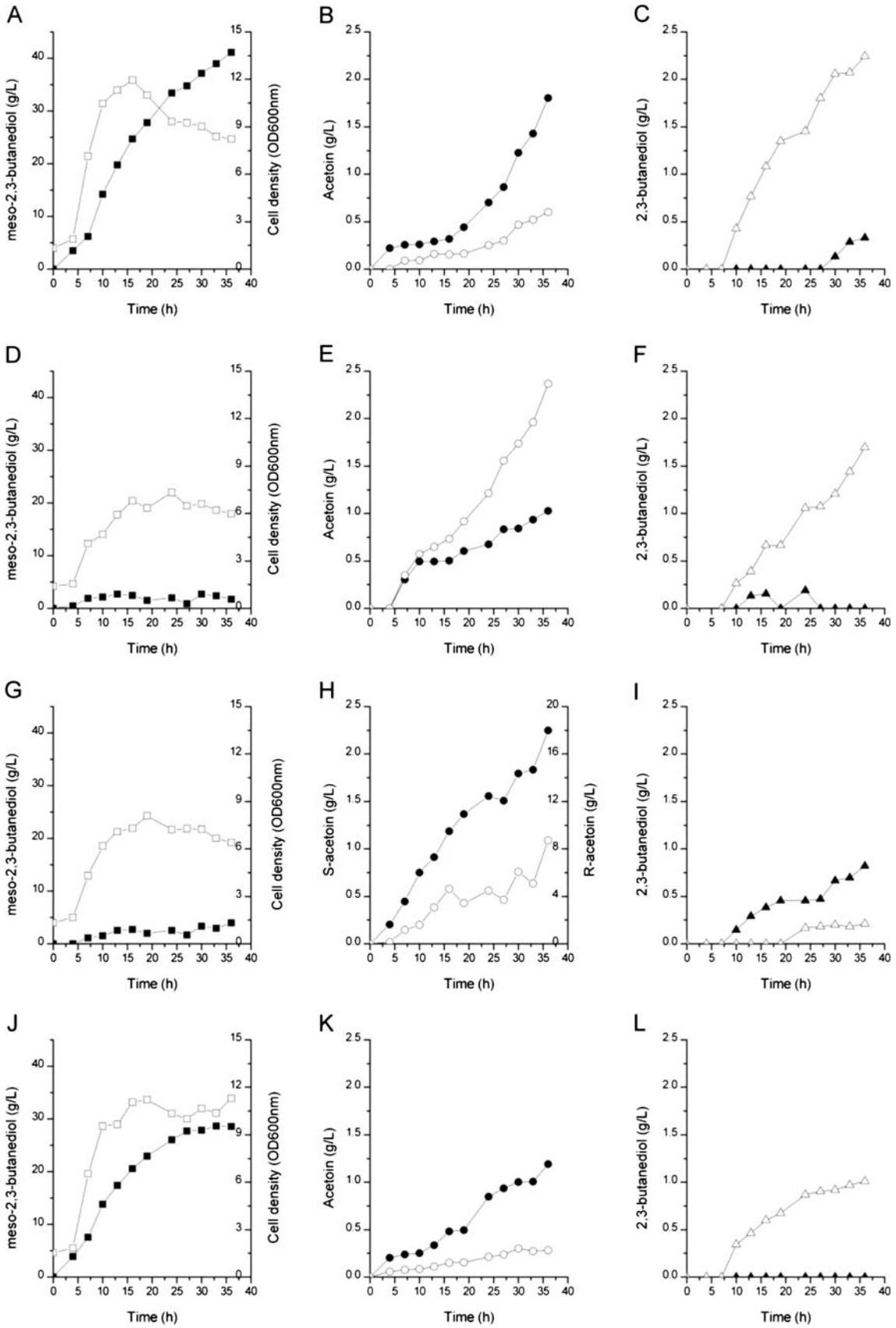
The main metabolic product of the wild-type strain was *meso*-2,3-butanediol when glucose was used as the carbon source. *Meso*-2,3-butanediol (41.1 g/L) was produced from a 36-h culture with a cell density of OD 8.3. Low levels of *R*-acetoin, *S*-acetoin, 2*S*,3*S*-butanediol, and 2*R*,3*R*-butanediol were produced at concentrations of 1.8, 0.6, 2.2, and 0.3 g/L, respectively. The cell growth of *K. pneumoniae* ΔbudA was slower than that of the wild-type strain, and the *meso*-2,3-butanediol produced was 2.0 g/L. The concentrations of *R*-acetoin, *S*-acetoin, and 2*S*,3*S*-butanediol produced were 1.0, 2.3, and 1.6 g/L, respectively. No 2*R*,3*R*-butanediol accumulated in the final broth, and only a low level of 2*R*,3*R*-butanediol appeared from 12 to 24 h. The growth of *K. pneumoniae* ΔbudC was also slower than that of the wild-type strain, and the concentration of *meso*-2,3-butanediol produced was reduced to 3.0 g/L. The concentrations of *S*-acetoin, 2*S*,3*S*-butanediol, and 2*R*,3*R*-butanediol produced were 1.0, 0.2, and 0.8 g/L, respectively. *R*-acetoin accumulated to a very high level (18 g/L). The cell growth of *K. pneumoniae* ΔdhaD was similar to that of the wild-type strain, and 28.6 g/L of *meso*-2,3-butanediol was produced. The amounts of *R*-acetoin, *S*-acetoin, and 2*S*,3*S*-butanediol produced were 1.2, 0.3, and 1.0 g/L, respectively. No 2*R*,3*R*-butanediol was produced by this mutant.

The wild-type strain exhibited *meso*-2,3-butanediol dehydrogenase-specific activity from 10 to 16 U/mg protein when glucose was used as the carbon source (Fig. 3). The activities in *K. pneumoniae* ΔbudA and *K. pneumoniae* ΔdhaD decreased to 3–7 and 8–13 U/mg protein, respectively. The *meso*-2,3-butanediol dehydrogenase activity of *K. pneumoniae* ΔbudC exhibited only 0.02–0.1 U/mg protein. The four strains showed low levels of glycerol dehydrogenase activities in the culture process of 0.01–0.09 U/mg protein.

Metabolic characteristics of *K. pneumoniae* ΔbudA, *K. pneumoniae* ΔbudC, and *K. pneumoniae* ΔdhaD using glycerol as the carbon source

The three mutants and the wild-type strain were cultured with glycerol as the carbon source. Figures 4 and 5 show the fermentation and enzyme activity results, respectively.

1,3-Propanediol was the main product of the cultures when glycerol was used as the carbon source, and concentrations of 1,3-propanediol at 59.1, 16.3, 53.3, and 21.0 g/L were produced by the wild-type strain, *K. pneumoniae* ΔbudA, *K. pneumoniae* ΔbudC, and *K. pneumoniae* ΔdhaD, respectively. Given that 1,3-propanediol is not related to butanediol isomer synthesis, its concentrations are not shown in the figure. The cell growth of the four stains was similar. The 2,3-butanediol isomers produced were different from those obtained using glucose as the carbon source. The wild-type strain *K. pneumoniae* CGMCC 1.6366 produced 14.5 g/L *meso*-2,3-butanediol, 1.0 g/L 2*S*,3*S*-butanediol, 5.5 g/L 2*R*,3*R*-butanediol, 0.18 g/L *S*-acetoin, and 0.27 g/L *R*-acetoin. The amounts of *S*-acetoin and *R*-acetoin produced by *K. pneumoniae* ΔbudA were similar to those of the wild-type strain, at concentrations of 0.20 and 0.27 g/L. The concentrations of 2,3-butanediol isomers produced by *K. pneumoniae* ΔbudA were all low at 0.9, 0.3, and 0.2 g/L for *meso*-2,3-butanediol, 2*S*,3*S*-butanediol, and 2*R*,3*R*-butanediol, respectively. *Meso*-2,3-butanediol (2.9 g/L) was produced by *K. pneumoniae* ΔbudC, and the amount of produced *S*-acetoin was similar to that from the wild-type strain at 0.16 g/L. Similar to the culture grown with glucose as the carbon source, this strain accumulated *R*-acetoin in the broth. However, the final concentration was 1.2 g/L, a value lower than that obtained using glucose as the carbon source. A distinguishing characteristic of this mutant was the production of a very high level of 2*R*,3*R*-butanediol (19.0 g/L). However, no 2*S*,3*S*-butanediol was produced by this strain. *K. pneumoniae* ΔdhaD produced 1.2 g/L *meso*-2,3-butanediol, 0.06 g/L *S*-acetoin, and 0.15 g/L *R*-acetoin. No 2*S*,3*S*-butanediol and 2*R*,3*R*-butanediol were produced by this mutant.


A
meso-2,3-butanediol (g/L)
Cell density (OD600nm)
Time (h)
B
Acetoin (g/L)
Time (h)
C
2,3-butanediol (g/L)
Time (h)
D
meso-2,3-butanediol (g/L)
Cell density (OD600nm)
Time (h)
E
Acetoin (g/L)
Time (h)
F
2,3-butanediol (g/L)
Time (h)
G
meso-2,3-butanediol (g/L)
Cell density (OD600nm)
Time (h)
H
S-acetoin (g/L)
R-acetoin (g/L)
Time (h)
I
2,3-butanediol (g/L)
Time (h)
J
meso-2,3-butanediol (g/L)
Cell density (OD600nm)
Time (h)
K
Acetoin (g/L)
Time (h)
L
2,3-butanediol (g/L)
Time (h)

◀ **Fig. 2** Metabolic products of *K. pneumoniae* CGMCC 1.6366 and three mutants using glucose as the carbon source. *Meso*-2,3-butanediol (*filled square*), cell density (*empty square*), *R*-acetoin (*filled circle*), *S*-acetoin (*empty circle*), 2*R*,3*R*-butanediol (*filled triangle*), and 2*S*,3*S*-butanediol (*empty triangle*). **a**–**c** Metabolic products of *K. pneumoniae* CGMCC 1.6366. **d**–**f** Metabolic products of *K. pneumoniae* ΔbudA. **g**–**i** Metabolic products of *K. pneumoniae* ΔbudC. **j**–**l** Metabolic products of *K. pneumoniae* ΔdhaD

The wild-type strain had a *meso*-butanediol dehydrogenase activity of 4–9 U/mg protein, which is about half of that using glucose as the carbon source. All three mutants had a very low level of *meso*-butanediol dehydrogenase activity. The wild-type strain and the ΔbudC mutant had a high level of glycerol dehydrogenase activity, about 10 to 20 times higher than that using glucose as the carbon source. *K. pneumoniae* ΔbudA had a glycerol dehydrogenase activity of 0.1–0.3 U/mg protein, which is also 10 times higher than that using glucose as the carbon source. *K. pneumoniae* ΔdhaD had a low glycerol dehydrogenase activity compared with that using glucose as the carbon source, with a value of 0.01–0.04 U/mg protein.

Stereospecific characteristics of butanediol dehydrogenase and glycerol dehydrogenase

E. coli BL21/budC and *E. coli* BL21/dhaD were constructed as described in "Materials and methods." *budC* and *dhaD* were heterologously expressed in *E. coli*, and the purified butanediol dehydrogenase and glycerol dehydrogenase were obtained using an AKTA purification system.

The stereospecific characteristics of the two enzymes were studied by catalysis reactions using diacetyl and acetoin as substrates. The results are shown in Fig. 6, and the specific activities of the two enzymes are listed in Table 3.

When diacetyl was used as the substrate, *R*-acetoin was the product of the butanediol dehydrogenase-catalyzed reaction. Neither *R*-acetoin nor *S*-acetoin was produced in the glycerol dehydrogenase-catalyzed reaction, though diacetyl was consumed in the reaction mixture. When racemic acetoin was used as the substrate, *meso*-2,3-butanediol and 2*S*,3*S*-butanediol were products of the butanediol dehydrogenase-catalyzed reaction, whereas *meso*-2,3-butanediol and 2*R*,3*R*-butanediol were products of the glycerol dehydrogenase-catalyzed reaction. *Meso*-2,3-butanediol was the main product of the two reactions. *Meso*-2,3-butanediol was derived from *R*-acetoin and *S*-acetoin in the presence of butanediol dehydrogenase and glycerol dehydrogenase, respectively.

Glycerol is the native substrate of glycerol dehydrogenase, but it is not a good substrate for butanediol dehydrogenase. Diacetyl and acetoin are the native substrates of butanediol dehydrogenase and are accepted as substrates for glycerol dehydrogenase. When diacetyl was used as the substrate, no acetoin was detected in the glycerol dehydrogenase reaction. However, a lower NADH consumption rate was observed.

Discussion

When glucose was used as the carbon source, the main metabolic product of the wild-type strain *K. pneumoniae* CGMCC 1.6366 was *meso*-2,3-butanediol together with low levels of 2*S*,3*S*-butanediol and 2*R*,3*R*-butanediol. The molar ratio of *meso*-2,3-butanediol, 2*S*,3*S*-butanediol, and 2*R*,3*R*-butanediol was 124:7:1. The production of 2*R*,3*R*-butanediol by *K. pneumoniae* has not been reported elsewhere perhaps because a low ratio of 2*R*,3*R*-butanediol is present compared with the other isomers. When glycerol was used as the carbon source, *K. pneumoniae* CGMCC 1.6366 produced all the 2,3-butanediol isomers, with *meso*-butanediol as the highest constituent. At a ratio of 2.6:0.18:1 for *meso*-2,3-butanediol, 2*S*,3*S*-butanediol, and 2*R*,3*R*-butanediol, the amount of

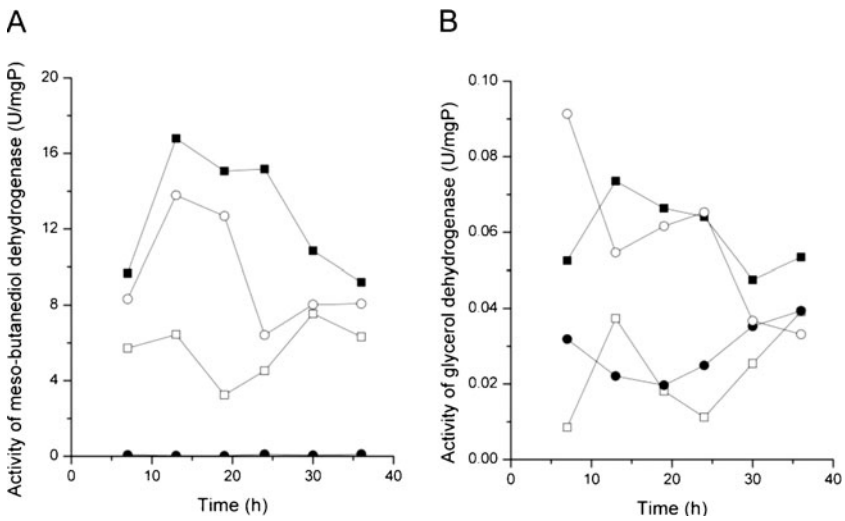


Fig. 3 *Meso*-2,3-butanediol dehydrogenase and glycerol dehydrogenase activities of the four strains with glucose as the carbon source. *K. pneumoniae* CGMCC 1.6366 (*filled square*), *K. pneumoniae* ΔbudA (*empty square*), *K. pneumoniae* ΔbudC (*filled circle*), and *K. pneumoniae* ΔdhaD (*empty circle*)

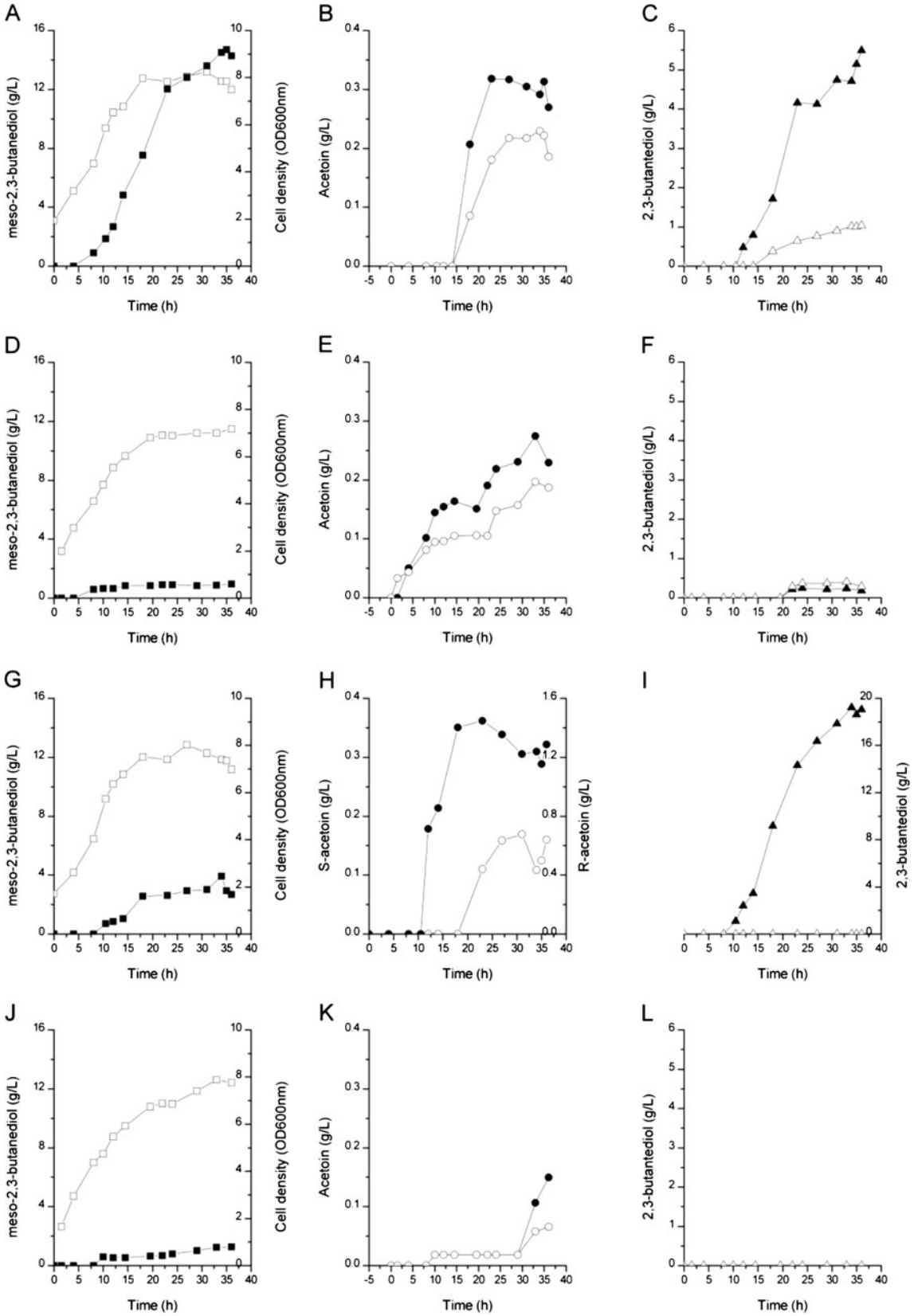


Fig. 4 Metabolic products of *K. pneumoniae* CGMCC 1.6366 and three mutants using glycerol as the carbon source. *Meso*-2,3-butanediol (*filled square*), cell density (*empty square*), *R*-acetoin (*filled circle*), *S*-acetoin (*empty circle*), 2*R*,3*R*-butanediol (*filled triangle*), and 2*S*,3*S*-butanediol (*empty triangle*). **a**–**c** Metabolic products of *K. pneumoniae* CGMCC 1.6366. **d**–**f** Metabolic products of *K. pneumoniae* ΔbudA. **g**–**i** Metabolic products of *K. pneumoniae* ΔbudC. **j**–**l** Metabolic products of *K. pneumoniae* ΔdhaD

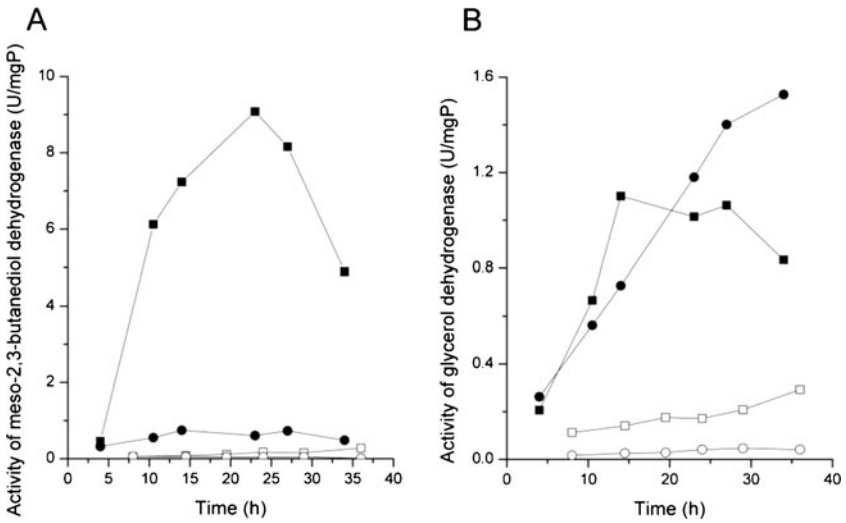


Fig. 5 *Meso*-2,3-butanediol dehydrogenase and glycerol dehydrogenase activities of the four strains with glycerol as the carbon source. *K. pneumoniae* CGMCC 1.6366 (*filled square*), *K. pneumoniae* ΔbudA (*empty square*), *K. pneumoniae* ΔbudC (*filled circle*), and *K. pneumoniae* ΔdhaD (*empty circle*)

2*R*,3*R*-butanediol concentration compared with the other isomers was increased. This result led us to hypothesize that the enzymes involved in glycerol metabolism are related to 2*R*,3*R*-butanediol formation. In vitro experiments showed that glycerol dehydrogenase catalyzed the reaction of *R*-acetoin and *S*-acetoin reduction to 2*R*,3*R*-butanediol and *meso*-2,3-butanediol, respectively. The *dhaD* mutant lost the ability to produce 2*R*,3*R*-butanediol when glucose or glycerol was used

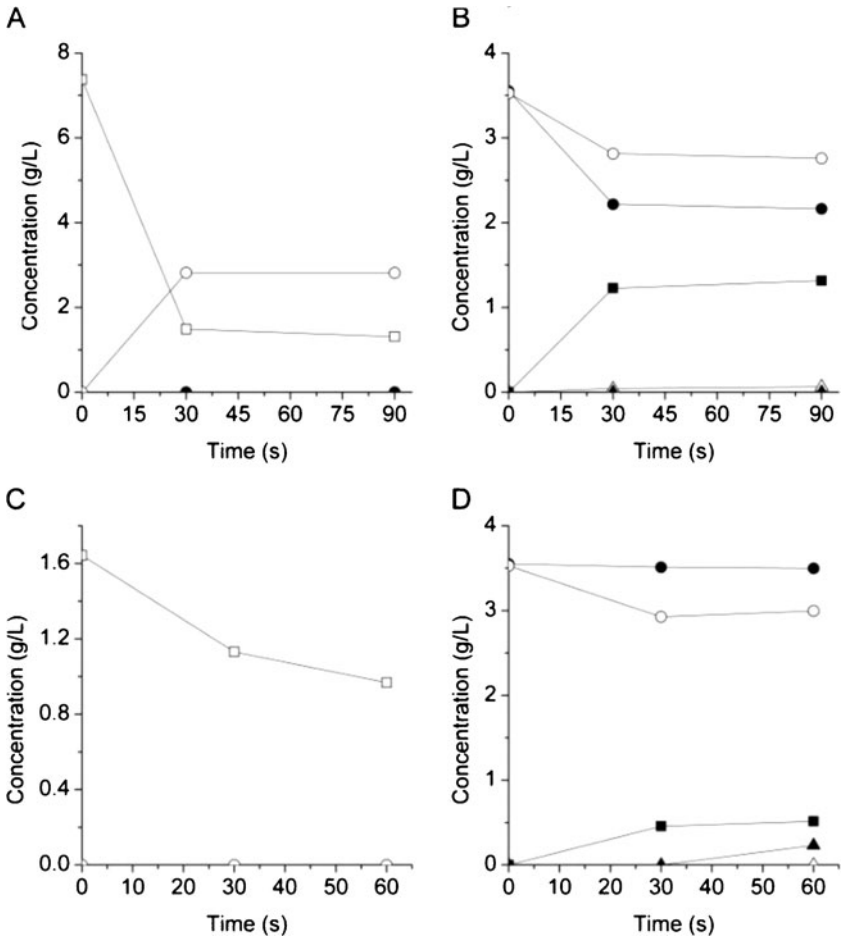


Fig. 6 Catalysis reactions of butanediol dehydrogenase and glycerol dehydrogenase. **a**, **b** Butanediol dehydrogenase. **c**, **d** Glycerol dehydrogenase. Diacetyl (*empty square*), *R*-acetoin (*filled circle*), *S*-acetoin (*empty circle*), *meso*-2,3-butanediol (*filled square*), 2*R*,3*R*-butanediol (*filled triangle*), and 2*S*,3*S*-butanediol (*empty triangle*)

Table 3 Activities of butanediol dehydrogenase and glycerol dehydrogenase using different substrates

Substrates	Butanediol dehydrogenase (U/mg protein)	Glycerol dehydrogenase (U/mg protein)
Glycerol (oxidation reaction)	0.01	6.70
Diacetyl	25.65	0.75
Racemic acetoin	10.33	5.91
R-acetoin	8.92	2.68

as the carbon source. Thus, glycerol dehydrogenase was the only candidate enzyme possible to contain 2*R*,3*R*-butanediol dehydrogenase activity in *K. pneumoniae*.

Glycerol dehydrogenase encoded by *dhaD* belongs to the short-chain dehydrogenase reductase family, which accepts a broad range of substrates. 1,2-Propanediol is commonly used in the enzyme activity assay (Forage and Foster 1982). This enzyme has high (*R*)-enantioselectivity for ketone reduction. 2*R*,3*R*-Butanediol is the main metabolite of some gram-positive bacterium. Up to 98 % of optically pure 2*R*,3*R*-butanediol can be produced by *Paenibacillus polymyxa* and *Bacillus licheniformis* (Gao et al. 2010; Häßler et al. 2012; Wang et al. 2012). The gene encoding 2*R*,3*R*-butanediol dehydrogenase in *P. polymyxa* was cloned, and the in vitro experiments showed that *R*-acetoin and *S*-acetoin can be used as substrates of this enzyme to form 2*R*,3*R*-butanediol and *meso*-butanediol (Gao et al. 2013). This stereospecificity is the same as that shown by the cloned glycerol dehydrogenase. However, there is no significant sequence homology between these two genes.

The *budC* mutant lost the ability to produce 2*S*,3*S*-butanediol, and the level of *meso*-butanediol exhibited a significant decrease. This phenotype indicates that 2*S*,3*S*-butanediol formation depends on the activity of *budC*. In vitro enzyme reactions showed that butanediol dehydrogenase can catalyze the conversion of diacetyl to *S*-acetoin and further to 2*S*,3*S*-butanediol as well as *R*-acetoin to *meso*-butanediol. Diacetyl is a nonenzymatic catalyzed product of α-acetolactate (Xiao and Xu 2007). Thus, butanediol dehydrogenase encoded by *budC* is the only known enzyme that catalyzes the conversion of diacetyl to *S*-acetoin and further to 2*S*,3*S*-butanediol.

budC is the only butanediol dehydrogenase-encoding gene that has been cloned from *K. pneumoniae*. This enzyme catalyzes the conversion of *R*-acetoin to *meso*-butanediol and has been used in engineering *E. coli* for *meso*-butanediol production (Lee et al. 2012). Zhang et al. (2012) showed that the butanediol dehydrogenase encoded by *budC* can use 2*S*,3*S*-butanediol as the substrate, but the special activity is lower than that obtained using *meso*-butanediol as the substrate. This enzyme showed no activity toward 2*R*,3*R*-butanediol, which is consistent with the results of the present study. Another butanediol dehydrogenase from *Rhodococcus erythropolis* shows a similar stereospecificity, and the activities shown toward 2*S*,3*S*-butanediol and *meso*-butanediol as substrates are of the same level (Wang et al. 2013). This enzyme and another butanediol dehydrogenase cloned from *Enterobacter cloacae* ssp. *dissolvens* can use diacetyl as the substrate to produce 2*S*,3*S*-butanediol through an *S*-acetoin intermediate (Li et al. 2012). The stereospecific conversion of the ketone to the *S*-form hydrogen group is the same as that obtained from the reaction catalyzed by the butanediol dehydrogenase cloned from *K. pneumoniae* CGMCC 1.6366.

K. pneumoniae ΔbudC still produced low amounts of *meso*-butanediol, suggesting that other enzymes exist in the cell which may contribute to the conversion of *R*-acetoin and/or *S*-acetoin to *meso*-butanediol. An in vitro experiment showed that glycerol dehydrogenase catalyzed the reaction of *S*-acetoin conversion to *meso*-butanediol. Therefore, glycerol dehydrogenase contributes to the *meso*-butanediol produced in *K. pneumoniae* ΔbudC.

The *budC* mutant accumulated high levels of *R*-acetoin in the culture using glucose as the carbon source and high levels of 2*R*,3*R*-butanediol in the culture using glycerol as the carbon source. The main metabolic pathway of *R*-acetoin use is converse to *meso*-butanediol. This pathway was blocked by *budC* gene mutation; thus, *R*-acetoin accumulated in the broth. When glycerol was used as the carbon source, the cells had a high glycerol dehydrogenase activity which catalyzed the conversion of *R*-acetoin to 2*R*,3*R*-butanediol. This mutant strain can be used for *R*-acetoin production because the 2*R*,3*R*-butanediol concentration is far lower than the *R*-acetoin level when glucose is used as the carbon source. The use of glycerol as the carbon source resulted in a high level of 2*R*,3*R*-butanediol accumulation. By contrast, only a small amount of *R*-acetoin was present, so this mutant can also be used for 2*R*,3*R*-butanediol production.

In the *K. pneumoniae* butanediol synthesis pathway, the acetoin produced from α-acetolactate was *R*-acetoin, not the *S*-form, which was confirmed by heterologous expression in *E. coli* (Ui et al. 1998). The *budA* mutant blocked the conversion of α-acetolactate to *R*-acetoin, but diacetyl can be produced without an influence from α-acetolactate. *K. pneumoniae* ΔbudA produced a higher amount of *S*-acetoin and a lower amount of *R*-acetoin than the wild-type strain when cultured using glucose as the carbon source. This result indicates that *S*-acetoin formation was not from *R*-acetoin, and the model that postulates the presence of an acetoin racemase reported by Voloch et al. (1983) is incorrect. Based on the phenotype of the three mutants and the in vitro enzyme reaction experiments, the metabolic pathway of 2,3-butanediol synthesis in *K. pneumoniae* is shown in Fig. 1.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (grant no. 20906076).

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