J Ind Microbiol Biotechnol (2015) 42:1105–1115
DOI 10.1007/s10295-015-1638-1

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BIOENERGY/BIOFUELS/BIOCHEMICALS

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R-acetoin accumulation and dissimilation in *Klebsiella pneumoniae*

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Received: 11 April 2015 / Accepted: 28 May 2015 / Published online: 10 June 2015
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Abstract *Klebsiella pneumoniae* is a 2,3-butanediol producer, and *R*-acetoin is an intermediate of 2,3-butanediol production. *R*-acetoin accumulation and dissimilation in *K. pneumoniae* was studied here. A *budC* mutant, which has lost 2,3-butanediol dehydrogenase activity, accumulated high levels of *R*-acetoin in culture broth. However, after glucose was exhausted, the accumulated *R*-acetoin could be reused by the cells as a carbon source. Acetoin dehydrogenase enzyme system, encoded by *acoABCD*, was responsible for *R*-acetoin dissimilation. *acoABCD* mutants lost the ability to grow on acetoin as the sole carbon source, and the acetoin accumulated could not be dissimilated. However, in the presence of another carbon source, the acetoin accumulated in broth of *acoABCD* mutants was converted to 2,3-butanediol. Parameters of *R*-acetoin production by *budC* mutants were optimized in batch culture. Aerobic culture and mildly acidic conditions (pH 6–6.5) favored *R*-acetoin accumulation. At the optimized conditions, in fed-batch fermentation, 62.3 g/L *R*-acetoin was produced by *budC* and *acoABCD* double mutant in 57 h culture, with an optical purity of 98.0 %, and a substrate conversion ratio of 28.7 %.

Keywords *R*-acetoin · Acetoin dehydrogenase enzyme system · 2,3-Butanediol dehydrogenase · *Klebsiella pneumoniae*

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Introduction

Acetoin (3-hydroxy-2-butanone) exists naturally in dairy products and some fruits, and commercial acetoin is used mainly as an additive to enhance the flavor of food. Some other applications have been reported, such as use in cosmetic products, as a feedstock for synthesis of other chemicals, as a chelating agent, and as a plant growth-promoting molecule [24]. Acetoin can be chemically synthesized from diacetyl (2,3-butanedione) [2], 2,3-butanediol [7], or acetaldehyde [1], and enzymatically synthesized from diacetyl [4], or 2,3-butanediol [11]. Acetoin can be produced by some microorganisms using glucose and other biomass-derived sugars as substrates, and has been considered to be one of the top 30 value-added chemicals from biomass [4].

Acetoin is an important metabolic product of microorganisms. The main physiological function of acetoin excretion is energy storage, and acetoin can be used as sole carbon source for the growth of some bacteria [25]. Acetoin 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 *R*-acetoin by α-acetolactate decarboxylase. *R*-acetoin can be further reduced to *meso*-2,3-butanediol by butanediol dehydrogenase. Genes encoding these enzymes are in the *bud* regulon and are regulated by BudR in *Klebsiella* spp. and other Gram-negative bacteria [17]. In *Bacillus* spp. and other Gram-positive bacteria, the regulon is named *als* and the AlsR is the transcriptional regulator [25]. 2*R*,3*R*-butanediol is synthesized from *R*-acetoin, employing glycerol dehydrogenase as the catalyzer in *Klebsiella pneumoniae* [3].

Acetoin dissimilation occurs in bacteria via an oxidative cleavage process which is catalyzed by an acetoin dehydrogenase enzyme system with acetaldehyde and acetyl

coenzyme A as the end products. The acetoin dehydrogenase enzyme system consists of thiamine PPi-dependent acetoin dehydrogenase (AoDH E1), dihydrolipoamide acetyltransferase (AoDH E2), and dihydrolipoamide dehydrogenase (AoDH E3), encoded by *acoA* (encoding the α subunit of AoDH E1), *acoB* (encoding the β subunit of AoDH E1), *acoC,* and *acoD*, respectively [9]. Theses genes are commonly located in *aco* gene clusters, and the expression of the *aco* operon is regulated by AcoK in *K. pneumoniae* [8]. The pathways of acetoin synthesis and use in *K. pneumoniae* are shown in Fig. 1.

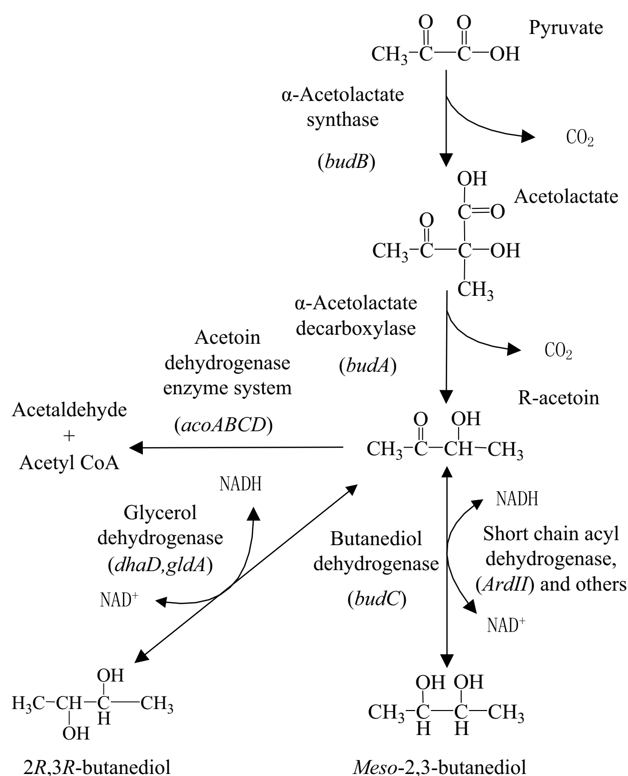

Fig. 1 Acetoin synthesis and use pathways in *K. pneumoniae*

Lactic acid bacteria and yeast have the capacity to produce acetoin as a flavor compound in fermented dairy products. However, acetoin is only produced by these microorganisms at a very low level [10, 15, 16]. Effective acetoin producers are bacteria including *Bacillus* spp., *Paenibacillus* spp., and *Serratia* spp. Some fermentation results are shown in Table 1.

Acetoin is chiral and has two enantiomers. However, the chirality of acetoin produced by fermentation is seldom described. Heterogeneous expression of the *bud* operon from *S. marcescens* in *E. coli* produced *R*-acetoin, with an optical purity of 97.3 % [26].

K. pneumoniae CGMCC 1.6366 (TUAC01) was isolated for 1,3-propanediol production [6]. In the investigation of 2,3-butanediol stereoisomer formation by this strain, we found that the *budC* mutant that had lost 2,3-butanediol dehydrogenase activity accumulated *R*-acetoin in the broth [3]. Hereby, *R*-acetoin accumulation and dissimilation in *K. pneumoniae* was investigated in details, and the *R*-acetoin production by the *budC* mutant and *budC-acoABCD* double mutant was investigated in batch and fed-batch fermentation.

Materials and methods

Strains, plasmids, and primers

Bacterial strains and plasmids used in this paper are listed in Table 2.

Construction of *K. pneumoniae* Δ*aco* and *K. pneumoniae* Δ*budC*–Δ*aco*

For mutant construction, *K. pneumoniae* and *E. coli* were grown in Luria–Bertani (LB) medium at 37 °C.

Table 1 Acetoin production by microorganisms

Strains	Fermentation	Acetoin (g/L)	Productivity (g/Lh)	Conversion ratio (g/g)	References
Bacillus licheniformis MEL09	Batch	41.3	1.15	0.41	[12]
Bacillus subtilis CICC 10025	Batch	35.4	0.63	0.28	[23]
Bacillus subtilis JNA-UD-6 (2,3-Butanediol dehydrogenase mutant)	Fed-batch	53.9	0.37	0.36	[30]
Bacillus subtilis (*bdhA* mutant and heterogenous expression of a water-forming NADH oxidase)	Batch	56.7	0.67	0.38	[29]
Escherichia coli (Heterogenous expression of *bud* operon)	Fed-batch	60.3	1.55	0.42	[26]
Paenibacillus polymyxa CS107	Fed-batch	55.3	1.32	0.37	[28]
Serratia marcescens H32 (Sucrose as carbon source)	Fed-batch	60.5	1.44	0.3	[19]
Serratia marcescens H32 (Heterologous expression of a water-forming NADH oxidase)	Fed-batch	75.2	1.88	0.36	[18]

Table 2 Strains and plasmids used in this work

Strains or plasmids	Relevant genotype and description	References or sources
Strains		
E. coli DH5α	Host of plasmid	Lab stock
K. pneumoniae CGMCC 1.6366	Wild-type	[6]
K. pneumoniae Δ*aco*	*K. pneumoniae* CGMCC 1.6366, Δ*acoABCD*	This work
K. pneumoniae Δ*budC*	*K. pneumoniae* CGMCC 1.6366, Δ*budC*	[3]
K. pneumoniae Δ*budC*–Δ*aco*	*K. pneumoniae* CGMCC 1.6366, Δ*budC*, Δ*acoABCD*	This work
Plasmid		
pDK6-red	Kan^r, carries λ-Red genes (gam, bet, exo)	[22]
pMD18-T-Δ*aco*	Amp^r, carries Δ*acoABCD*	This work
pMD18-T-*aco*	Amp^r, carries *acoABCD*	This work
pIJ773	$Apra^r$, *aac*(*3*)*IV* with FRT sites	[5]

The antibiotics used in selective medium were ampicillin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}), and apramycin (50 μg mL^{-1}).

K. pneumoniae Δ*aco* was constructed according to a previously described method [22]. In brief, *acoABCD* was amplified from the genome of *K. pneumoniae* CGMCC 1.6366 using the primer pair aco-s1 (GCCTGGTGA CCTTTATTGAGGAGAA)/aco-a1 (ACTGAAATGTCGG CATCCGCAAAGC). The PCR product was then ligated into pMD18-T Simple to generate pMD18-T-*aco*. Linear DNA with 39 and 40 bp homologous extensions flanking the apramycin resistance gene, *aac*(*3*)*IV*, was amplified from plasmid pIJ773 using the primer pair aco-s2 (TCTTT GAAGCCATCAATATGGCCGTCGTGCTCCAGCTC CATTCCGGGGATCCGTCGACC)/aco-a2 (CGGCAAGG ATCGCGCTTTCCAGATCAGAAACAGAGATAC GTGTAGGCTGGAGCTGCTTC). pMD18-T-Δ*aco* was constructed by replacing part of *acoABCD* in plasmid pMD18-T-aco with the *aac*(*3*)*IV* cassette using the Red recombinase system in *E. coli*. pMD18-T-Δ*aco* was then used as a template for PCR preparation of linear DNA containing *aac*(*3*)*IV* with long homologous regions at either end. The primers used were aco-s1 and aco-a1. Finally, the linear DNA was transformed into *K. pneumoniae* CGMCC 1.6366 which already host pDK6-red. Homologous recombination between linear DNA and the chromosome was mediated by Red recombinase. *K. pneumoniae* Δ*aco* was isolated on apramycin plates, and the primer pair Test773 (GCAAATACGGCATCAGTTACC) and aco-s1 was used for PCR confirmation of the mutant. In this process, part of *acoA*, whole *acoB,* and part of *acoC* in the chromosome of *K. pneumoniae* were replaced by the mark gene.

K. pneumoniae Δ*budC*–Δ*aco* was constructed following the same method as *K. pneumoniae* Δ*aco*, with *K. pneumoniae* Δ*budC* replacing *K. pneumoniae* CGMCC 1.6366 as the target strain.

Acetoin use

Basic medium was used for acetoin use tests and contained (g/L): racemic acetoin, 4; Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; $MgSO_4·7H_2O$, 0.5; and $GaCl_2·6H_2O$, 0.5. *Klebsiella pneumoniae* CGMCC 1.6366 and mutants were inoculated in tubes that contained 3 mL medium and cultured aerobically at 37 °C overnight.

Fermentation medium and culture condition

Fermentation medium contained (g/L): glucose, 100; yeast extract, 2; corn steep liquor, 4; $(NH_4)_2SO_4$, 5; sodium acetate, 3; KCl, 0.4; and $MgSO_4$, 0.1.

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. 50 mL of seed culture were inoculated into a 5-L bioreactor (BIOSTAT-A plus Sartorius) with a working volume of 3 L. Culture temperature was kept at 37 °C. The culture pH was automatically controlled by the addition of 1 M NaOH and HCl aqueous solutions. In fed-batch culture, a bottle of glucose solution (100 g glucose and 50 g water) was fed to the bioreactor when the glucose level in the medium decreased to 5 g/L.

Optimization of the culture parameters

Culture parameters including oxygen supplementation and culture pH were studied in batch fermentation. A different oxygen supplementation in the fermentation process was achieved by controlled agitation. The air supplement was 4 L/min, and the agitation was set at 300, 400, 500, and 700 rpm, respectively. In this experiment, the culture pH was kept stable at 6. For experiments to study the effect of culture pH, the air supplementation and the stirring agitation were maintained at 4 L/min and 500 rpm, respectively. The culture pH was set at pH 5.5, 6.0, 6.5, or 7.0.

Analytical methods

Biomass concentration was evaluated by determination of optical density (OD) at 600 nm with a NanoDrop-2000C spectrophotometer (Thermo-Scientific, Wilmington, DE). Chemical components in the broth such as glucose, acetoin, *meso*-2,3-butanediol, 2*R*,3*R*-butanediol, 2-ketogluconic acid, succinic acid, acetic acid, and ethanol were quantified by a Shimadzu 20AVP high-performance liquid chromatography system (Shimadzu Corp., Kyoto, Japan) with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad, USA) and a 0.005 mol/L H_2SO_4 solution at 0.8 mL/min flow rate. Chiral isomers of acetoin and 2,3-butanediol in the broth were quantified using a gas chromatography (GC) system (Shimadzu GC 2010) equipped with a flame ionization detector and a Rt®-bDEXse column, as previously described [3].

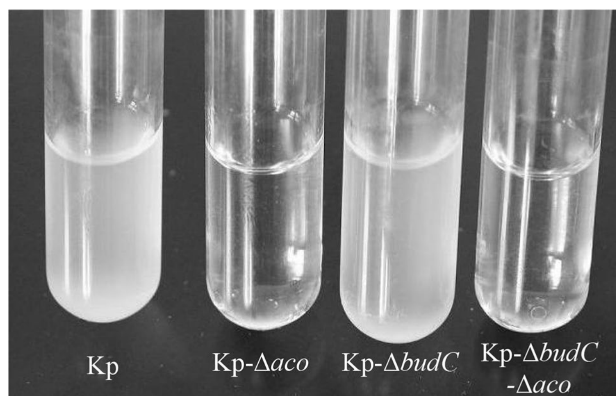

Fig. 2 Growth of wild-type and *acoABCD*-mutated strains of *K. pneumoniae* with acetoin as the sole carbon source. Kp: *K. pneumoniae* CGMCC 1.6366; Kp-Δ*aco*: *K. pneumoniae* Δ*aco*; Kp-Δ*budC*: *K. pneumoniae* Δ*budC*; Kp-Δ*budC*-Δ*aco*: *K. pneumoniae* Δ*budC*–Δ*aco*

Results

Acetoin dehydrogenase enzyme system mutation

The acetoin dehydrogenase enzyme system encoding genes *acoABCD* was knocked out in *K. pneumoniae* CGMCC 1.6366 and *K. pneumoniae* Δ*budC* to construct *K. pneumoniae* Δ*aco* and *K. pneumoniae* Δ*budC*–Δ*aco*, respectively. The four strains were cultured in basic medium with acetoin as the sole carbon source. Figure 2 shows the growth of these strains. *K. pneumoniae* CGMCC 1.6366 and *K. pneumoniae* Δ*budC* grew to turbidity. However, *K. pneumoniae* Δ*aco* and *K. pneumoniae* Δ*budC*–Δ*aco* did not grow. The wild-type strain can use acetoin as the sole carbon source for growth, but the *acoABCD* mutants have lost that ability.

Acetoin accumulation and dissimilation in *K. pneumoniae* Δ*budC*

K. pneumoniae Δ*budC* was cultured in batch to produce acetoin. Figure 3 shows the results of the culture process.

The whole process of batch fermentation was divided to three phases. Phase I was from the inoculation to the exhaust of glucose, during this period, cells consume glucose for rapid growth, and 28 g/L acetoin was produced. Lower levels of *meso*-2,3-butanediol, 2*R*,3*R*-butanediol, lactic acid, succinic acid, and ethanol were produced. Phase II was from 16 to 24 h culture. During this period, organic acids and ethanol produced in the broth were reused by the cells, and the acetoin level also decreased to about 26 g/L. Phase III was the remaining time of the culture. Acetoin was consumed at a higher speed than that in the phase II. The final metabolic products were *meso*-2,3-butanediol and 2*R*,3*R*-butanediol, with the concentration of 10.7 and 2.6 g/L, respectively.

The effect of the acetoin dehydrogenase enzyme system on acetoin accumulation

K. pneumoniae Δ*budC*–Δ*aco* was cultured in batch to produce acetoin compared to *K. pneumoniae* Δ*budC*. The results were shown in Fig. 3.

During phase I and II, cell growth and metabolites production of *K. pneumoniae* Δ*budC*–Δ*aco* were the same as that of *K. pneumoniae* Δ*budC*. However, cell growth, acetoin consumption, *meso*-2,3-butanediol, and 2*R*,3*R*-butanediol production were all stopped during phase III.

The effect of other carbon source supplement on acetoin dissimilation

As about 2 g/L acetoin was consumed by *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco* during phase II. At the same time, ethanol, succinic acid, and lactic acid produced in the broth were all used by the cell. After these chemicals were exhausted, acetoin consumption in *K. pneumoniae* Δ*budC*–Δ*aco* was stopped. We suspected that acetoin dissimilation during this period was the result of ethanol, succinic acid, and lactic acid used by the cell as carbon source and supply NADH to cells.

To test this hypothesis, *K. pneumoniae* Δ*budC*–Δ*aco* was cultured again and extra ethanol was regularly added to the culture in phase III (Fig. 3). When this extra ethanol was supplied, the acetoin level decreased continuously and the *meso*-2,3-butanediol and 2*R*,3*R*-butanediol levels

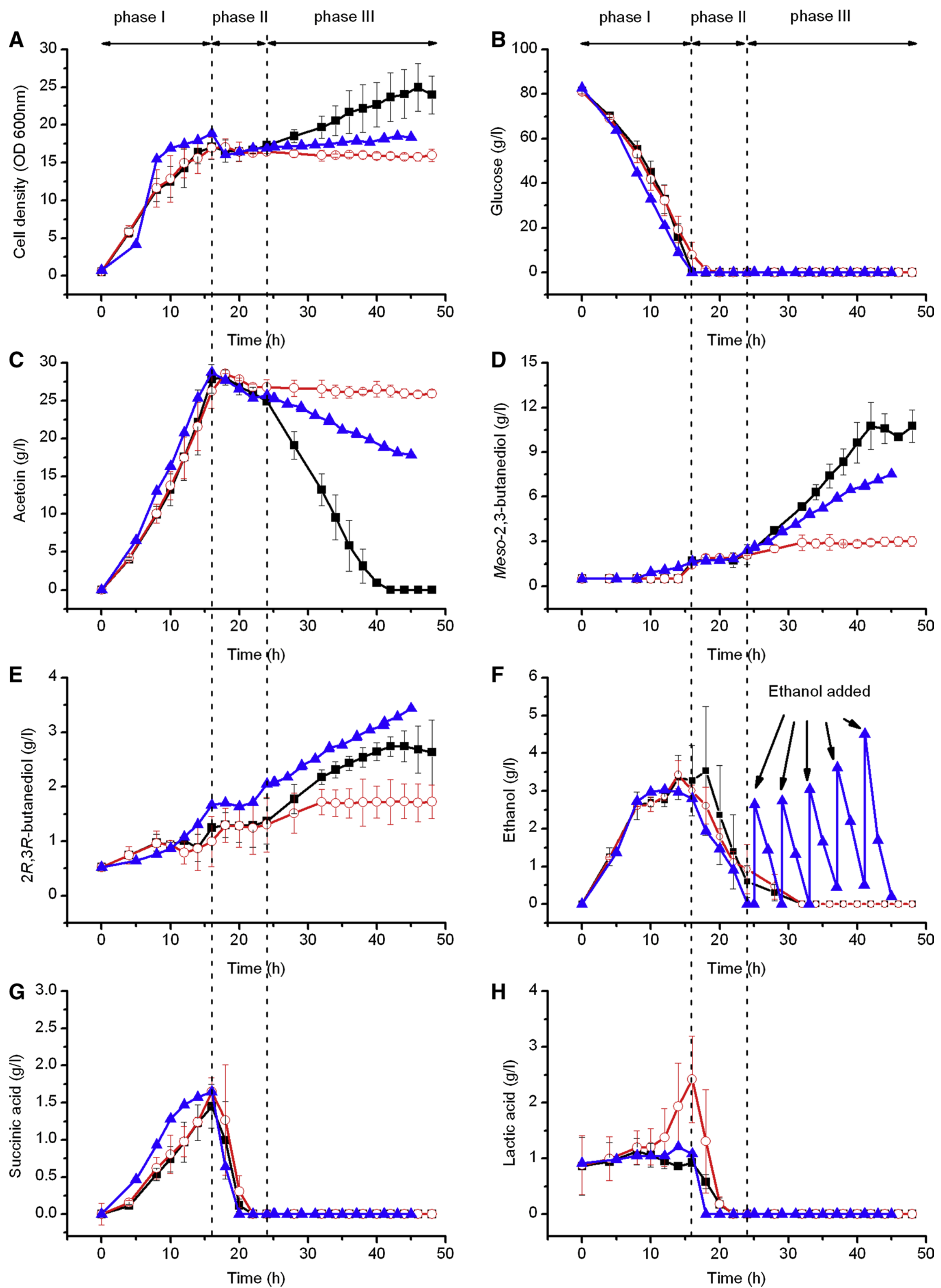

Fig. 3 Batch culture of *K. pneumoniae* Δ*budC* (*filled square*), *K. pneumoniae* Δ*budC*-Δ*aco* (*unfilled circle*), and *K. pneumoniae* Δ*budC*-Δ*aco* with outside ethanol added (*filled triangle*)

increased continuously. However, the acetoin consumption rate was slower than that of *K. pneumoniae* Δ*budC*.

The effect of oxygen supplementation on acetoin production

As acetoin production was not obviously different between *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco* in the culture before glucose exhaustion, *K. pneumoniae* Δ*budC* was used for culture parameter optimization. In the bioreactor, oxygen supplementation has a positive relationship with agitation. Different agitations were used to study the effect of oxygen supplementation on acetoin production; Fig. 4 shows the fermentation results.

Figure 4a and b shows that cell growth and glucose consumption improved with increased agitation. Glucose was exhausted quickest with agitation at 700 rpm, followed by 500 and 400 rpm. Cell growth was slowest with agitation at 300 rpm, and 50 g/L glucose remained in the broth after culture for 16 h. As well as the slowest growth, 300 rpm agitated culture had the lowest acetoin level. Acetoin levels in 400 rpm and 500 rpm cultures were similar to each other. The culture agitated at 700 rpm had similar acetoin productivity as those at 400 and 500 rpm. However, the conversion ratio was lower (Fig. 4c), owing to accumulation of 2-ketogluconic acid, which is an intermediate of glucose metabolism. The higher the oxygen supplementation, the more 2-ketogluconic acid accumulated (Fig. 4d). *Meso*-2,3-butanediol production was similar for different agitations and at very low levels in each case (Fig. 4e). Ethanol production had a negative relationship with oxygen supplementation and the 300 rpm agitated culture had the highest ethanol level (Fig. 4f). Succinic acid production had a positive relationship with oxygen supplementation, thus the 700 rpm culture had the highest succinic acid level. Lactic acid is another byproduct of acetoin production; 400 and 500 rpm agitated cultures had the highest lactic acid level. Based on the results in Fig. 4, agitation at 400–500 rpm was the optimum value for culture oxygen supplementation.

The effect of culture pH on acetoin production

The effect of culture pH on acetoin production by *K. pneumoniae* Δ*budC* was investigated and fermentation results are shown in Fig. 5. In the pH range of 5.5–7.0, cell growth and glucose consumption improved with increasing pH (Fig. 5a, b). The highest level of acetoin was achieved at pH 6, while the highest productivity was at pH 6.5 (Fig. 5c). 2-Ketogluconic acid accumulation showed a negative relationship with culture pH, lower pH favoring 2-ketogluconic acid accumulation (Fig. 5d). However, 2-ketogluconic acid accumulated in the broth could be reused by the cells. *Meso*-2,3-butanediol was only produced at low levels and followed the same tendency as acetoin production (Fig. 5e). Ethanol production had a positive relationship with culture pH; culture at pH 7 resulted in the highest ethanol level, 5.2 g/L (Fig. 5F). Succinic acid and lactic acid production had a positive relationship with culture pH; lower pH inhibited the organic acid production (Fig. 5g, h). Considering the productivity and final acetoin level, a culture pH of 6.0–6.5 was the optimum value.

Acetoin production in fed-batch fermentation

Acetoin production by *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco* was studied in fed-batch fermentation. The agitation and culture pH were set at 500 rpm and pH 6.5, respectively. Figure 6 shows the fermentation results.

Cell growth of the two strains was similar. Cells grew quickly in the first 12 h and reached the highest density at 16–21 h, after which there was some decrease (Fig. 6a). Acetoin production at a high rate coincided with the rapid cell growth phase. After that phase, acetoin was produced at a lower rate. Acetoin production was faster in *K. pneumoniae* Δ*budC*–Δ*aco* than in *K. pneumoniae* Δ*budC* in the first 40 h of culture; 61.0 and 57.4 g/L acetoin were produced by the two strains, respectively. However, the final acetoin levels, after 57 h, were similar; 61.9 and 62.3 g/L for *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco*, respectively (Fig. 6b). The total calculated conversion ratios of glucose to acetoin were 27.9 ± 1.7 and 28.7 ± 1.1 %, respectively. Cells begin synthesis of *meso*-2,3-butanediol and 2*R*,3*R*-butanediol after 9 h of culture, after which their concentrations increased almost linearly. The final concentrations of *meso*-2,3-butanediol and 2*R*,3*R*-butanediol produced by *K. pneumoniae* Δ*budC* were 14.8 and 7.1 g/L, and by *K. pneumoniae* Δ*budC*–Δ*aco* were 14.1 and 7.2 g/L, respectively, (Fig. 6c, d). Ethanol was produced in the rapid cell growth phase and was consumed after that phase (Fig. 6e). Lactic acid was mainly synthesized from 12 to 21 h of culture and after that it was reused by the cells (Fig. 6g). Succinic acid and acetic acid were mainly synthesized after cells stopped growing; unlike ethanol and lactic acid, they were not consumed by the cells (Fig. 6f, h).

Samples of *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco* broth at the end of the culture period were analyzed by GC to test for enantiomers of acetoin and 2,3-butanediol. Table 3 shows the results.

GC analysis showed that the acetoin produced by the two strains was mainly *R*-acetoin. The optical purity of *R*-acetoin produced by *K. pneumoniae* Δ*budC*–Δ*aco* was 98.0 %. There was no 2*S*,3*S*-butanediol produced by the two strains.

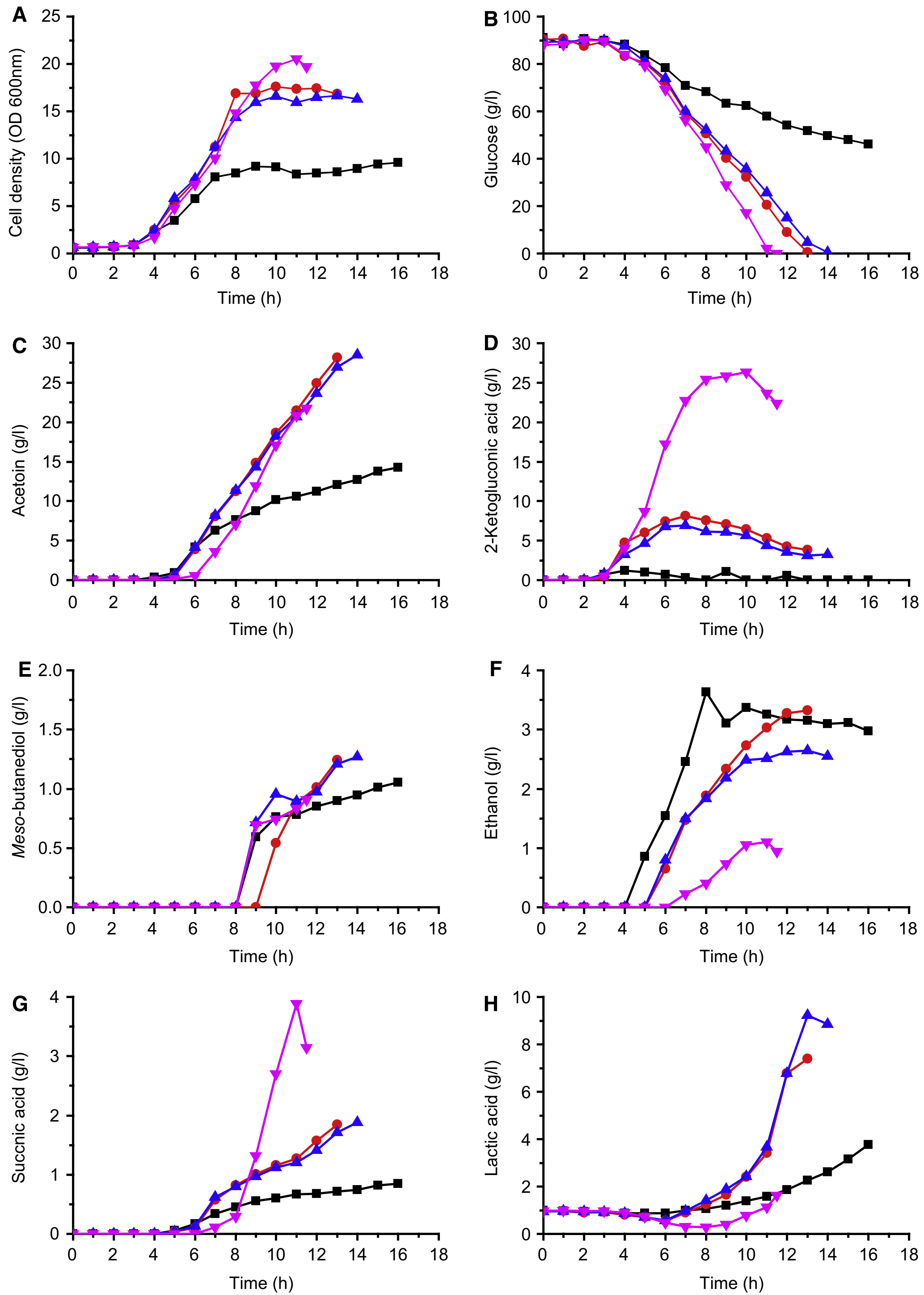

Fig. 4 The effect of oxygen supplementation on acetoin production by *K. pneumoniae* Δ*budC*. Agitation of cultures at 300 rpm (*filled square*); 400 rpm (*filled circle*); 500 rpm (*filled triangle*); or 700 rpm (*filled inverted triangle*)

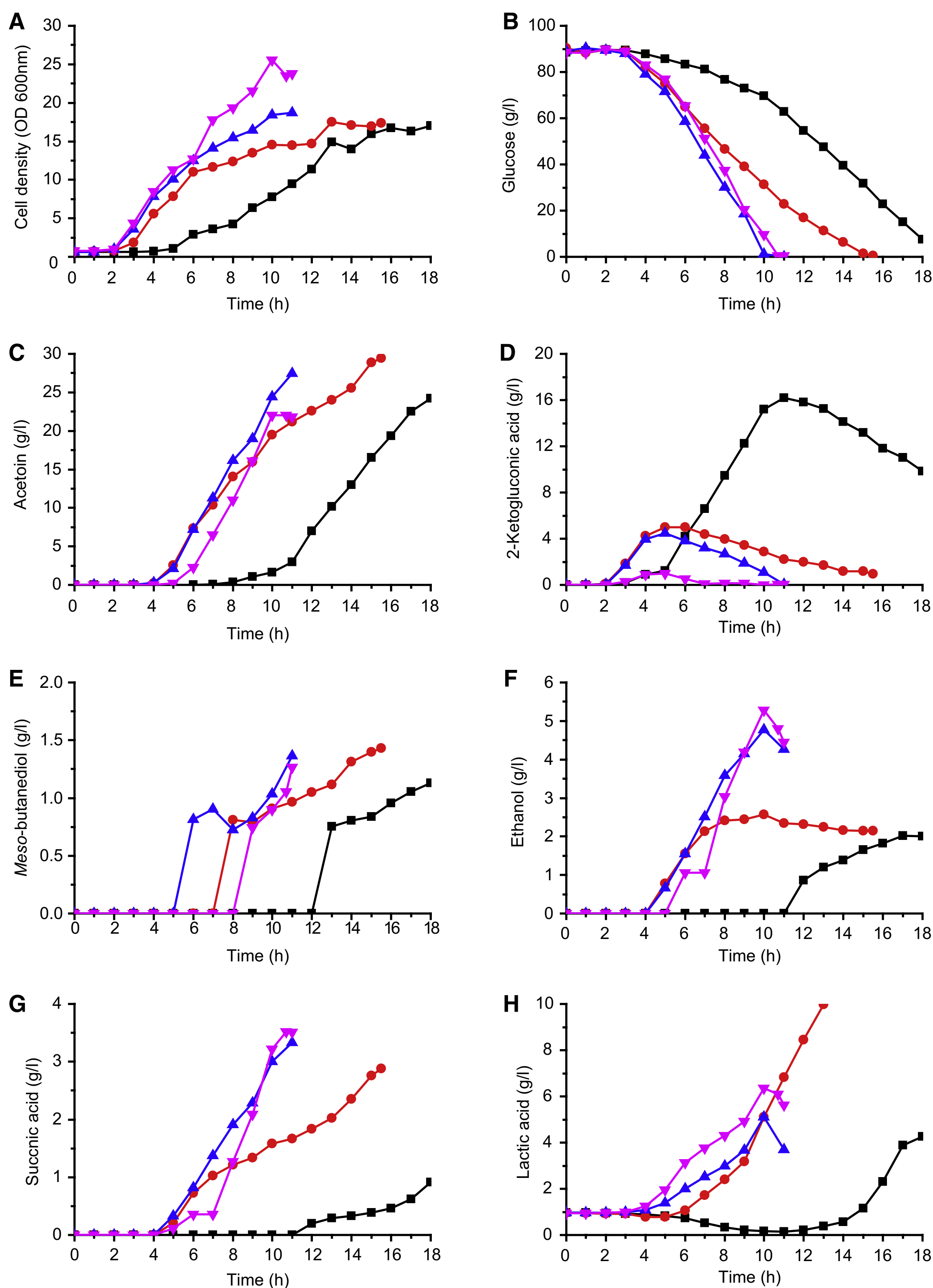

Fig. 5 The effect of culture pH on acetoin production. pH 5.5 (*filled square*); pH 6.0 (*filled circle*); pH 6.5 (*filled triangle*); pH 7.0 (*filled inverted triangle*)

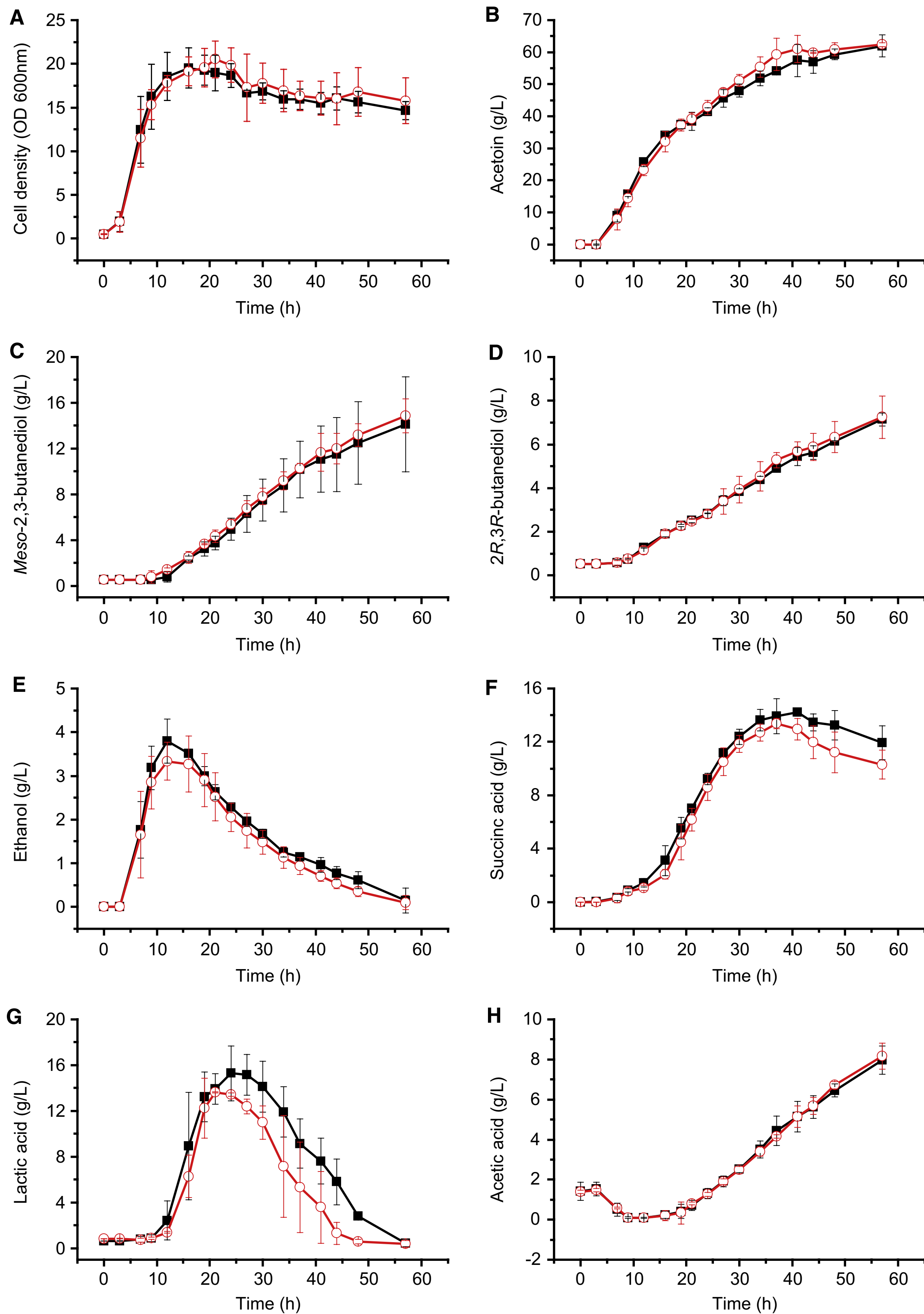

Fig. 6 Acetoin production in fed-batch fermentation. *K. pneumoniae* Δ*budC* (*filled square*) and *K. pneumoniae* Δ*budC*–Δ*aco* (*unfilled circle*)

Table 3 Enantiomers of acetoin and 2,3-butanediol produced in fed-batch fermentation

Strains	*R*-acetoin (g/L)	*S*-acetoin (g/L)	2*R*,3*R*-butanediol (g/L)	2*S*,3*S*-butanediol (g/L)
K. pneumoniae Δ*budC*	59.2 ± 0.6	1.3 ± 0.3	7.5 ± 1.0	0
K. pneumoniae Δ*budC*–Δ*aco*	64.1 ± 2.5	1.3 ± 0.8	6.7 ± 0.8	0

Discussion

Acetoin is an intermediate in the 2,3-butanediol synthesis pathway. Using glucose as carbon source, the main metabolic product of *K. pneumoniae* is 2,3-butanediol, accompanied by a low level of acetoin. Butanediol dehydrogenase mutation results in *R*-acetoin accumulation in culture broth [3]. Wild-type strains of *K. pneumoniae* can grow on basic medium using acetoin as the carbon source (Fig. 2), and the *R*-acetoin accumulated in the broth can be reused by the cells (Fig. 3). This is in agreement with the physiological function of acetoin as an energy-storing chemical [25]. *acoABCD* mutants, however, lost the ability to use acetoin as the carbon source for growth, indicating that oxidative cleavage catalyzed by the acetoin dehydrogenase enzyme system is the sole pathway of acetoin dissimilation in *K. pneumoniae*. Thus, the additional benefit of *K. pneumoniae* Δ*budC*–Δ*aco* is *R*-acetion produced during fermentation which can not be dissimilated by themselves.

In the batch culture of *K. pneumoniae* Δ*budC*–Δ*aco*, after glucose was exhausted, about 2 g/L acetoin was consumed. We considered that this fraction of the acetoin was converted to 2,3-butanediol. A possible explanation is that the *acoABCD* mutation did not affect the acetoin conversion to 2,3-butanediol, but this reaction requires coenzyme NADH. After glucose exhaustion, organic acids and ethanol were reused by the cells. Thus, in this period, *R*-acetoin in the broth can be converted to 2,3-butanediol with the NADH supplied by organic acid and ethanol catabolism. After the organic acids and ethanol were exhausted, no further acetoin was converted into 2,3-butanediol owing to a lack of NADH. The experiment where extra ethanol was added into the culture of *K. pneumoniae* Δ*budC*–Δ*aco* confirms this hypothesis. During phase II and phase III, 9 g/L 2,3-butanediol was produced as the cells consumed 11 g/L of acetoin, so most of acetoin consumed was converted to 2,3-butanediol. However, only 10 g/L 2,3-butanediol was synthesized by *K. pneumoniae* Δ*budC* cells at the price of 28 g/L acetoin. This indicated a fraction of acetoin was catabolized, like the extra supplemented ethanol, and supplied NADH for other fraction of acetoin conversion to 2,3-butanediol.

Meso-butanediol and 2*R*,3*R*-butanediol were the main byproducts in acetoin production by *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco*. The reason is that the other cellular dehydrogenases besides *budC*-encoded butanediol dehydrogenase catalyze the conversion of acetoin to 2,3-butanediol (Fig. 1). We have demonstrated that a *dhaD*-encoded glycerol dehydrogenase shows *R*-enantioselective butanediol dehydrogenase activity [3]. Another glycerol dehydrogenase encoded by *gldA* also catalyzed the conversion of acetoin to 2,3-butanediol [21]. A short-chain acyl dehydrogenase was identified in *K. pneumoniae*; this enzyme was an *S*-enantioselective butanediol dehydrogenase, like BudC [14]. 2,3-Butanediol isomers synthesis was also found in the *budC* mutant of *K. oxytoca* [27].

Aerobic conditions favor acetoin production. However, excess oxygen supplementation resulted in 2-ketogluconic acid accumulation. Furthermore, excess oxygen supplementation reduced the conversion of glucose to acetoin. It has been reported that expression of the *bud* operon was anaerobically induced [13], transcription of genes for acetoin production might be inhibited during aerobic growth of cells, so carbon flux was channeled to other chemicals. In research on acetoin production by *Paenibacillus polymyxa*, the effect of oxygen supplementation on acetoin production was similar; the highest acetoin level was obtained at a medium agitation [28].

Culture pH is another key parameter in fermentation and mildly acidic conditions favor acetoin synthesis. Beside anaerobiosis, low pH is another factor that induces *bud* operon expression in *K. terrigena* [13]. At neutral pH, a large part of the glucose was oxidized to CO_2, as in conditions of high oxygen supplementation. At pH 5.5, 2-ketogluconic acid accumulated, an acidic pH-dependent process [20].

In fed-batch fermentation, acetoin level increased quickly in a period of initial 40 h for both *K. pneumoniae* Δ*budC* and *K. pneumoniae* Δ*budC*–Δ*aco*; in extended fermentation, the ratio of *meso*-2,3-butanediol and 2*R*,3*R*-butanediol formation exceeded the acetoin formation, therefore, fermentation should be stopped after 40 h to achieve economical production of acetoin. Compared with acetoin produced by other microorganisms, *budC* mutants of *K. pneumoniae* are able to produce more *R*-acetoin which demonstrates higher optical purity. However, its substrate conversion ratio is rather low, probably due to the formation of 2,3-butanediol. Since all known acetoin producing bacteria also produce 2,3-butanediol as byproduct, with the only exception of a recombinant *E. coli* which was able to produce *R*-acetoin with very high substrate conversion rate by expressing a *bud* operon original from

Serratia marcescens [26]. It is also known that the substrate conversion ratio of acetoin from *Serratia marcescens* H32 increased as the level of 2,3-butanediol was decreasing [18, 19]. Therefore, lower the production of 2,3-butanediol through metabolic engineering should be a promising strategy for further improvement of *R*-acetoin productivity by *K. pneumoniae.*

budC mutants of *K. pneumoniae* are high *R*-acetoin-producing strains and have the potential to be used in industry. *acoABCD* mutants block the acetoin dissimilation pathway, and *R*-acetoin produced during fermentation can not be reused by the cell. *K. pneumoniae* Δ*budC*–Δ*aco* produced 62.3 g/L *R*-acetoin in fed-batch fermentation. This result is the highest level of *R*-acetoin produced from glucose by biological methods.

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

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