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# Microbial production of *meso*-2,3-butanediol by metabolically engineered *Escherichia coli* under low oxygen condition

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**Abstract** A metabolically engineered *Escherichia coli* has been constructed for the production of *meso*-2,3-butanediol (2,3-BD) under low oxygen condition. Genes responsible for 2,3-BD formation from pyruvate were assembled together to generate a high-copy plasmid pEnBD, in which each gene was transcribed with a constitutive promoter. To eliminate by-product formation under low oxygen condition, genes including *ldhA*, *pta*, *adhE*, and *poxB* which functioned for the mixed acid fermentation pathways were deleted in *E. coli* JM109. Compared with the wild type, the quadruple gene deletion mutant produced smaller amounts of acetate, succinate, and ethanol from glucose when cultivated in LB medium in shake flasks under low-aeration. When 2,3-BD producing pathway was introduced via pEnBD into the mutant, higher glucose consumption and faster 2,3-BD production rate compared with that of the wild-type control were observed under aerobic condition in shake flasks. In a 6-L fermentor supplied with only 3% dissolved oxygen (DO), the mutant harboring pEnBD converted glucose to 2,3-BD much faster than the control did. When DO supply was further lowered to 1% DO, the recombinant mutant grew much slower but produced 2,3-BD as a major fermentation metabolic product. In addition, the 2,3-BD yield showed an increase from 0.20 g BD/g glucose for the control to 0.43 g BD/g glucose for the mixed acid pathway deleted mutant grown in fermentors under 1% DO. These results reveals the potential of production of enantiomerically pure 2,3-BD isomer by recombinant *E. coli* under low oxygen condition.

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**Keywords** *meso*-2,3-butanediol · Microaerobic · Mixed acid fermentation · *Escherichia coli* · Metabolic engineering

## Introduction

2,3-butanediol (2,3-BD) is an important chemical feedstock and the production of 2,3-BD is receiving increasing attentions for its extensive industrial applications (Syu 2001; Celińska and Grajek 2009). 2,3-BD can be used as an antifreeze agent due to its low freezing point of −60°C. The dehydration of 2,3-BD generates the industrial solvent methyl ethyl ketone, which can be used as an effective fuel additive having a higher heat of combustion than that of ethanol. Further dehydration of 2,3-BD yields 1,3-butadiene, a substance used in synthetic rubber production (Syu 2001; Celińska and Grajek 2009). The dehydrogenation of 2,3-BD produces diacetyl, which is a valuable flavoring agent and bacteriostatic food additive for the food industry (Bartowsky and Henschke 2004).

2,3-BD exists in three stereoisomers, the *dextro*-, *levo*-, and *meso*-forms. Many bacterial species can ferment pyruvate to 2,3-BD by homologous pathways involving three enzymes: acetolactate synthase, acetolactate decarboxylase, and acetoin reducase (Fig. 1) (Syu 2001; Celińska and Grajek 2009). Depending on the microorganism and the cultivation conditions, the ratio of 2,3-BD stereoisomers can vary dramatically. *Bacillus subtilis* produces a mixture of *levo*- and *meso*-forms of 2,3-BD. *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Enterobacter aerogenes* produces *dextro*- and *meso*-stereoisomers of 2,3-BD (Celińska and Grajek 2009). In *Bacillus polymyxa*, the *levo*-form of 2,3-BD is produced as a major product, a purity of over 98% can be obtained from this wild-type strain (Hespell 1996; Nakashimada et

al. 1998). *K. pneumoniae*, *K. oxytoca*, and *B. polymyxa* are considered to be strong candidates for efficient production of 2,3-BD, and much efforts have been devoted to the optimization of growth conditions for maximal 2,3-BD productivity and yield (Beronio and Tsao 1993; Nakashimada et al. 1998; Ji et al. 2009a; Ji et al. 2009b; Ma et al. 2009). Furthermore, genetic modifications were also applied to eliminate by-product formation for enhanced 2,3-BD production (Ji et al. 2008; Ji et al. 2010).

Recombinant *Escherichia coli* possesses strong potentials for 2,3-BD production due to its advantages including the ability to utilize several inexpensive carbon sources, the ease for genetic manipulations, and the well-studied metabolic pathways. Moreover, the enantiomerically pure 2,3-BD isomers could be obtained by expressing the stereospecific enzymes. So far, enantiomerically pure *meso*-, *levo*-, and *detro*-forms of 2,3-BD were all obtained by engineered *E. coli* (Ui et al. 1997b; Ui et al. 2004; Yan et al. 2009). Oxygen supply is considered to be the most important factor for 2,3-BD fermentation by the native producers. It governs the distribution of fermentation end-products and controls the yield of 2,3-BD (Zeng et al. 1990; Beronio and Tsao 1993; Nakashimada et al. 1998; Converti et al. 2003; Celińska and Grajek 2009). However, in recombinant *E. coli* harboring 2,3-BD producing pathway, the effect of oxygen supply on 2,3-BD production has not yet been investigated carefully, and previous studies mainly focused on the aerobic production processes. 2,3-BD production under low oxygen or microaerobic condition can permit simple reactor design, easy control strategies, and convenient operation conditions, which would be an environmentally and economically sustainable process. In this study, we altered the mixed acid fermentation pathways of *E. coli* and studied the production of *meso*-2,3-butanediol under low oxygen condition (Fig. 1).

## Materials and methods

### Microorganisms and medium

The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* As 1.1700 was provided by China General Microbiological Culture Collection Center and used for *alsS* and *alsD* amplification. *K. pneumonia* 14sp-N was a gift from Prof. De-Hua Liu of Tsinghua University and used for *budC* cloning. *E. coli* JM109 was purchased from TaKaRa Bio Inc. (Shiga, Japan) and used as the cloning host for 2,3-BD production studies. Gene deletions in *E. coli* JM109 were performed according to the method reported previously (Li et al. 2010). Primers used to generate PCR fragments for gene knockout are listed in Table 2. All strains were cultivated in Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L Bacto tryptone, and 10 g/L NaCl).

### Plasmid construction

Standard procedures or manufacturers' instructions were used for molecular cloning experiments. Plasmid isolation and DNA purification kits were purchased from Qiagen (Shanghai, China). Restriction enzymes and DNA modifying enzymes were provided by MBI Fermentas (Vilnius, Lithuania). Table 2 lists primers used for gene cloning.

Plasmid pEn was used as the expression vector; it harbors the pyruvate decarboxylase promoter ($P_{pdc}$) of *Zymomonas mobilis*, multiple cloning sites, and the *rrnBT2* transcriptional terminator. For assembling genes in a stepwise manner, *Eco*RI-*Avr*II restriction sites were designed upstream of $P_{pdc}$, and *Nhe*I-*Xho*I restriction sites were designed downstream of *rrnBT2* (Li et al. 2010).

The *alsS* gene was amplified from *B. subtilis* genomic DNA using primers B1#F/B1#R and cloned into the *Bam*HI/*Sac*I sites of pEn to construct pEnB1. The *alsD* gene was cloned from *B. subtilis* genomic DNA using primers B2#F/B2#R and inserted into the *Bam*HI/*Sac*I sites of pEn to generate pEnB2. The *budC* gene was amplified from *K. pneumonia* genomic DNA using primers B5#F/B5#R and cloned into the *Bam*HI/*Sac*I sites of pEn to give pEnB5. To construct pEnAC, $P_{pdc}$-*alsD* was excised from pEnB2 using *Avr*II/*Xho*I and cloned into pEnB1 digested with *Nhe*I/*Xho*I. To construct pEnBD, $P_{pdc}$-*budC* was excised from pEnB5 using *Avr*II/*Xho*I and cloned into pEnAC digested with *Nhe*I/*Xho*I (Fig. 2).

### Culture conditions

The seed culture was grown at 37°C in LB medium for 12 h on a rotary shaker at 200 rpm. For shake flask cultures, seed culture (4%, *v*/*v*) was inoculated into LB medium supplemented with glucose as carbon source and cultivated at 37°C for 48 h. Fifty milliliters of culture broth in a 500-mL baffled flask was cultivated at 200 rpm to achieve a high-aeration condition, whereas 100 mL culture broth in a 250-mL no-baffled flask was cultivated at 100 rpm for the low-aeration condition.

For fermentor cultures, seed culture was inoculated into a 6-L fermentor (NBS 3000, New Brunswick, USA) at 10% inoculation volume with an operating volume of 3 L. The fermentation medium contained (grams per liter): glucose, 60; yeast extract, 10; Bacto tryptone, 10; and NaCl, 10. For the low oxygen cultivation, dissolved oxygen (DO) was provided by injecting filtered air at a flow rate of 1.5 L/min and was maintained at 3% or 1% of air saturation by automatically adjusting the agitation rate from 200 to

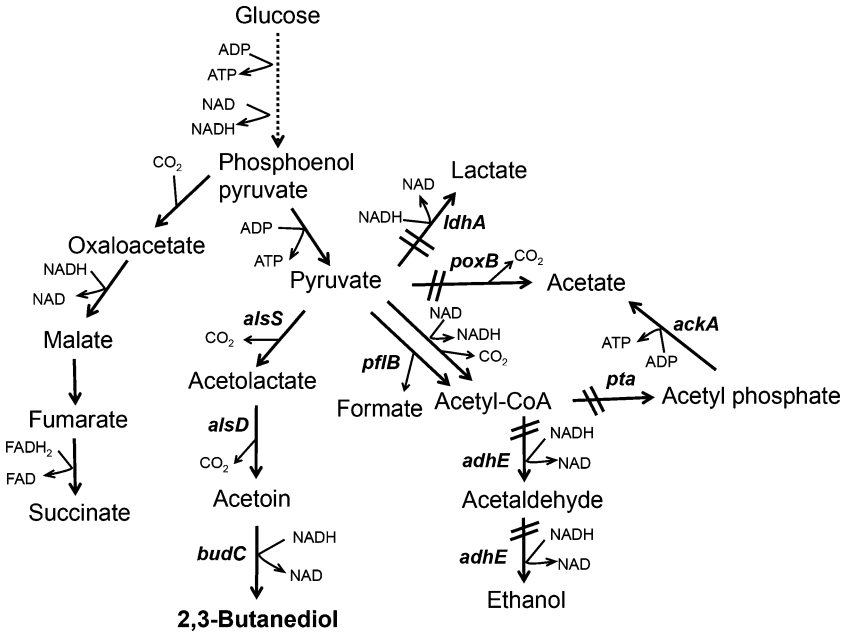



**Fig. 1** *meso*-2,3-butanediol production pathway in *Escherichia coli*. Genes: *alsS* acetolactate synthase, *alsD* acetolactate decarboxylase, *budC* acetoin reducase, *ldhA* D-lactate dehydrogenase, *pta* phosphate acetyltransferase, *ackA* acetate kinase, *adhE* acetaldehyde dehydrogenase/alcohol dehydrogenase, *poxB* pyruvate oxidase, *pflB* pyruvate formate lyase

500 rpm. The pH in the fermentor culture was maintained at 6.8 by automatic addition of 5 M NaOH and 5 M $H_2SO_4$. In all cases, a final concentration of 100 μg/mL ampicillin was added to the medium to maintain the stability of plasmid pEnBD.

## Analytical methods

Bacteria were harvested by centrifugation at 8,000×*g* for 10 min and then washed with distilled water. The supernatant was filtered through a 0.2-μm syringe filter

**Table 1** Strains and plasmids used in this study

| Strains/plasmids | Description | Reference/source |
|---|---|---|
| *Bacillus subtilis* As 1.1700 | Source of *alsS* and *alsD* | CGMCC |
| *Kebsiella pneumonia* 14sp-N | Source of *budC* | Lab stock |
| *E. coli* JM109 | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)*/F′[*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15] | TaKaRa Bio Inc. |
| JM109L | *E. coli* JM109 *ΔldhA* | This study |
| JM109LP | *E. coli* JM109 *ΔldhA Δpta* | This study |
| JM109LPA | *E. coli* JM109*ΔldhA Δpta ΔadhE* | This study |
| JM109LPAP | *E. coli* JM109*ΔldhA Δpta ΔadhE ΔpoxB* | This study |
| pKD13 | Template plasmid with Kan resistance gene and FLP recognition target | (Datsenko and Wanner 2000) |
| pCP20 | FLP recombinase helper plasmid, *ts-rep*, $Amp^R$, $Cm^R$ | (Datsenko and Wanner 2000) |
| pEn | Expression plasmid, $P_{pdc}$, pMD19-T derived | (Li et al. 2010) |
| pKD46EnRecA | RecA and λ Red recombinase expression helper plasmid, *oriR101*, *repA101*(ts), $Amp^R$ | (Li et al. 2010) |
| pEnB1 | *alsS* inserted into pEn | This study |
| pEnB2 | *alsD* inserted into pEn | This study |
| pEnB5 | *budC* inserted into pEn | This study |
| pEnAC | $P_{pdc}$-*alsD* inserted into pEnB1 | This study |
| pEnBD | $P_{pdc}$-*budC* inserted into pEnAC | This study |

**Table 2** Oligonucleotides used in this study

| Primers | Sequence (5′-3′) |
|---|---|
| B1#F | GGATCCAAGGAGATATACCATGTTGACAAAAGCAACAAAAGAAC |
| B1#R | GAGCTCTTAGAGAGCTTTCGTTTTCATGA |
| B2#F | GGATCCAAGGAGATATACCATGAAACGAGAAAGCAACATTCAA |
| B2#R | GAGCTCTTATTCAGGGCTTCCTTCAGTTG |
| B5#F | GGATCCAAGGAGATATACCATGAAAAAAGTCGCACTTGTTACCG |
| B5#R | GAGCTCTTAGTTAAATACCATCCCGCCGTC |
| ldhAF | TATTTTTAGTAGCTTAAATGTGATTCAACATCACTGGAGAAAGTCTTATGGTGTAGGCTGGAGCTGCTTCG |
| ldhAR | CTCCCCTGGAATGCAGGGGAGCGGCAAGATTAAACCAGTTCGTTCGGGCAATTCCGGGGATCCGTCGACC |
| ptaF | GCTGTTTTGTAACCCGCCAAATCGGCGGTAACGAAAGAGGATAAACCGTGGTGTAGGCTGGAGCTGCTTCG |
| ptaR | GCAGCGCAAAGCTGCGGATGATGACGAGATTACTGCTGCTGTGCAGACTGATTCCGGGGATCCGTCGACC |
| adhEF | CGAGCAGATGATTTACTAAAAAAGTTTAACATTATCAGGAGAGCATTATGGTGTAGGCTGGAGCTGCTTCG |
| adhER | CCGTTTATGTTGCCAGACAGCGCTACTGATTAAGCGGATTTTTTCGCTTTATTCCGGGGATCCGTCGACC |
| poxBF | GATGAACTAAACTTGTTACCGTTATCACATTCAGGAGATGGAGAACCATGGTGTAGGCTGGAGCTGCTTCG |
| poxBR | CCTTATTATGACGGGAAATGCCACCCTTTTTACCTTAGCCAGTTTGTTTTATTCCGGGGATCCGTCGACC |

All oligonucleotides were synthesized by AuGCT Biotech (Beijing, China). Restriction endonuclease digestion sites were underlined

and stored chilled for high-performance liquid chromatography (HPLC) analysis. Cell dry weight was measured after vacuum lyophilization. The concentration of glucose, lactate, succinate, formate, acetate, 2,3-BD, acetoin, and ethanol was determined by HPLC (P2000, AS3000, Thermo Spectra System, USA), equipped with an ion exchange column (Aminex® HPX-87H, 7.8×300 mm, BioRad) and a refractive index detector (RI-150, Thermo Spectra System, USA). A mobile phase of 5 mM $H_2SO_4$ at a 0.5 mL/min flow rate was used.

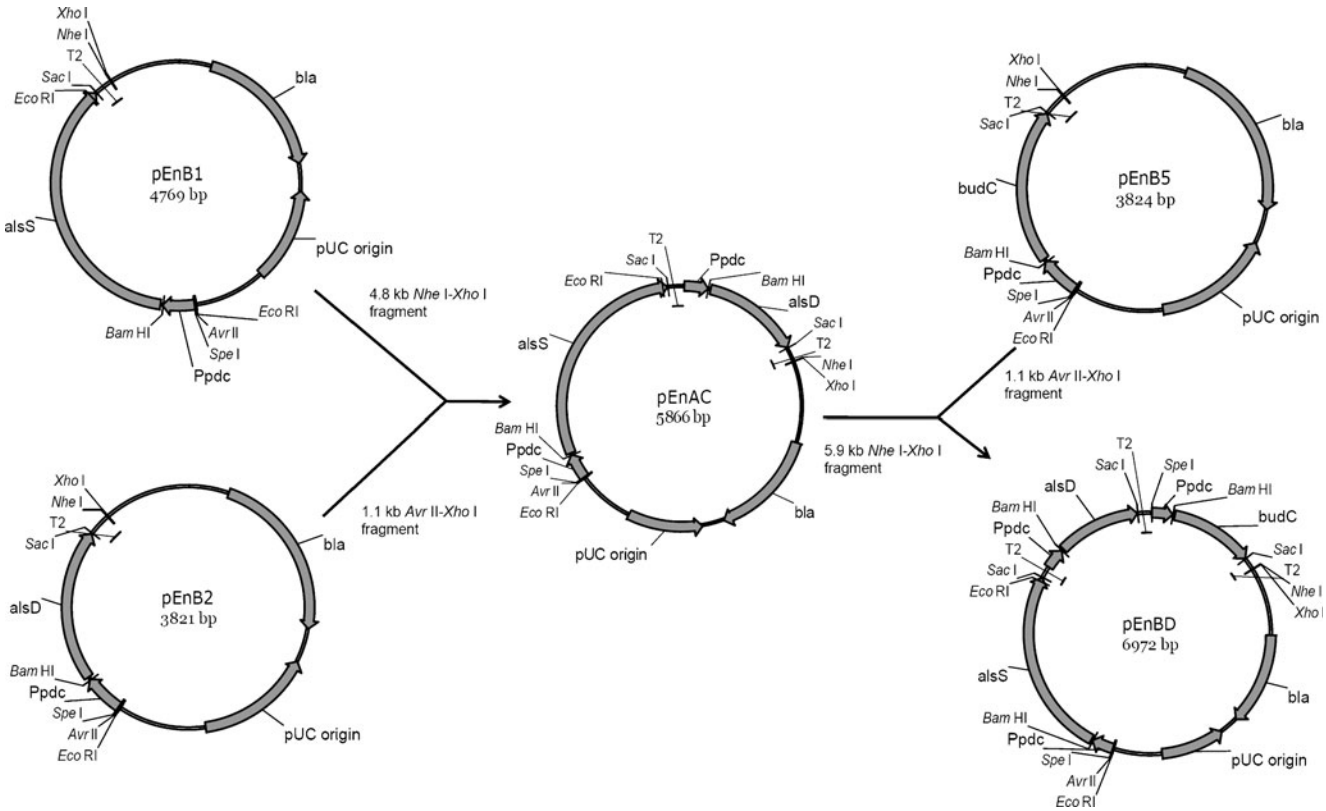



**Fig. 2** Structure of plasmids used in this study. See "Materials and methods" for descriptions on the construction of these plasmids

**Table 3** Cell dry weight, glucose consumption, and extracellular metabolite production of *E. coli* JM109 and its altered mixed acid fermentation pathway mutants in shake flasks

| Strains | CDW (g/L) | Glucose consumed (g/L) | Lactate (g/L) | Acetate (g/L) | Succinate (g/L) | Ethanol (g/L) | Formate (g/L) |
|---|---|---|---|---|---|---|---|
| JM109 | 0.81±0.01 | 3.42±0.08 | 0.02±0.00 | 2.84±0.01 | 0.04±0.00 | 0.18±0.01 | ND |
| JM109L | 1.29±0.03 | 4.50±0.06 | ND | 3.25±0.05 | 0.05±0.00 | 0.28±0.01 | ND |
| JM109LP | 1.35±0.03 | 4.40±0.04 | ND | 3.08±0.06 | 0.05±0.00 | 0.21±0.02 | ND |
| JM109LPA | 1.29±0.08 | 3.72±0.08 | ND | 2.92±0.04 | 0.05±0.00 | 0.10±0.01 | ND |
| JM109LPAP | 1.12±0.04 | 2.20±0.05 | ND | 0.83±0.01 | 0.11±0.00 | 0.07±0.01 | ND |

*E. coli* strains were cultivated in LB medium containing 2% (*w*/*v*) glucose under low-aeration condition at 37°C for 48 h as described in "Materials and methods". Data shown are the average and standard deviation of three parallel experiments

*CDW* cell dry weight, *ND* not detected

## Results

### Gene cloning and plasmid construction

Three enzymes are required for 2,3-BD production from pyruvate, including acetolactate synthase, acetolactate decarboxylase, and acetoin reducase. In *B. subtilis*, the *alsSD* operon encoding the acetolactate synthase and acetolactate decarboxylase, is responsible for acetoin production from pyruvate (Renna et al. 1993; Ramos et al. 2000). In *K. pneumonia*, *budC* gene encodes the acetoin reducase and catalyzes *meso*-2,3-butanediol formation from acetoin (Ui et al. 1997a; Wardwell et al. 2001). To achieve effective 2,3-BD production in *E. coli*, the genes mentioned above were cloned and inserted into the high-copy expression vector pEn to generate pEnB1, pEnB2, and pEnB5, respectively. Subsequently, the genes were assembled together by a stepwise manner to yield the final expression plasmid pEnBD. Under the designed gene assembly, each gene was driven by the constitutively transcribed promoter $P_{pdc}$, which ensured the high gene expression level for effective 2,3-BD production (Fig. 2).

### Disruption of mixed acid fermentation pathway in *E. coli* JM109

Under anaerobic conditions, *E. coli* strains use mixed acid fermentation for glucose metabolism to form the end-products including ethanol, succinate, lactate, acetate, formate, hydrogen, and carbon dioxide. Under oxygen limitation, the mixed acid fermentation also occurs. To eliminate the by-products formation during the 2,3-BD production process under low oxygen conditions, genes including *ldhA*, *pta*, *adhE*, and *poxB* were all deleted in *E. coli* JM109 by the improved Wanner's method reported previously (Li et al. 2010). The successful gene deletions were confirmed by both PCR verification and DNA sequencing.

Shake flask cultures were performed to evaluate effects of gene deletions on glucose metabolism. *E. coli* JM109 and its mutants were cultivated in LB medium supplemented with 20 g/L glucose under low-aeration condition at 37°C for 48 h as described in "Materials and methods" (Table 3). Among the five strains, the wild-type strain showed the lowest cell dry weight. The deletion of *ldhA* totally blocked lactate formation. Meanwhile, the *ldhA* mutant consumed more glucose and produced more acetate, succinate, and ethanol compared with what the parent strain did. Further deletion of *pta* in *ldhA* mutant resulted in a slight decrease for acetate production. The deletion of *adhE* obviously decreased glucose consumption and ethanol production, yet the ethanol formation was not totally eliminated. The deletion of *poxB* in JM109LAP caused a significant reduction on glucose consumption and acetate production, while an increase for succinate formation was observed.

### Production of meso-2,3-BD in shake flask cultures

Plasmid pEnBD was electro-transformed into *E. coli* JM109 and JM109LPAP, respectively, then the recombinants were cultivated in LB medium supplemented with glucose for 2,3-butanediol production. High and low oxygen conditions were employed in shake flask cultures. To achieve high-aeration condition, 50 mL culture broth in a 500-mL baffled flask was inoculated at 200 rpm, whereas 100 mL culture broth in a 250-mL non-baffled flask was cultivated at 100 rpm for the low-aeration condition. Under the high-aeration condition, the wild type and mutant harboring pEnBD showed similar cell dry weight, succinate and acetate producing profiles, yet higher glucose consumption and 2,3-BD production were observed in the mutant (Table 4). Under the low-aeration condition, glucose was consumed rapidly by both strains. However, *E. coli* JM109LPAP (pEnBD) exhibited faster growth rate and higher final cell dry weight. Moreover, the production of by-products including lactate, formate, and ethanol was

**Table 4** Cell dry weight, 2,3-BD production, glucose consumption, and extracellular metabolite production by *E. coli* strains cultivated under different oxygen conditions in shake flasks

| Strains | CDW (g/L) | Glucose consumed (g/L) | 2,3-BD (g/L) | Acetoin (g/L) | Lactate (g/L) | Acetate (g/L) | Succinate (g/L) | Ethanol (g/L) | Formate (g/L) | $Y_{BD+acetoin/glucose}$ | BD productivity (g/L/h) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| JM109[a] | 5.01±0.06 | 39.77±1.23 | 12.84±0.71 | 1.37±0.04 | ND | Trace | 0.10±0.00 | ND | ND | 0.36±0.01 | 0.27±0.01 |
| JM109LPAP[a] | 4.92±0.02 | 47.88±0.70 | 14.50±0.36 | 2.49±0.05 | ND | Trace | 0.09±0.00 | ND | ND | 0.35±0.01 | 0.30±0.01 |
| JM109[b] | 2.47±0.16 | 28.91±0.10 | 10.21±0.63 | 0.50±0.11 | 0.19±0.03 | Trace | 0.26±0.01 | 1.20±0.12 | 0.12±0.02 | 0.37±0.02 | 0.21±0.01 |
| JM109LPAP[b] | 3.05±0.04 | 28.53±0.50 | 9.46±0.83 | 2.32±0.86 | ND | Trace | 0.55±0.03 | 0.36±0.01 | ND | 0.41±0.01 | 0.20±0.02 |

Recombinant *E. coli* strains were cultivated in LB medium containing 5% (*w*/*v*; for high-aeration cultivation) or 3% (*w*/*v*) glucose (for low-aeration cultivation) at 37°C for 48 h as described in "Materials and methods". Data shown are the average and standard deviation of three parallel experiments

*CDW* cell dry weight, *ND* not detected

[a] High-aeration cultivation

[b] Low-aeration cultivation

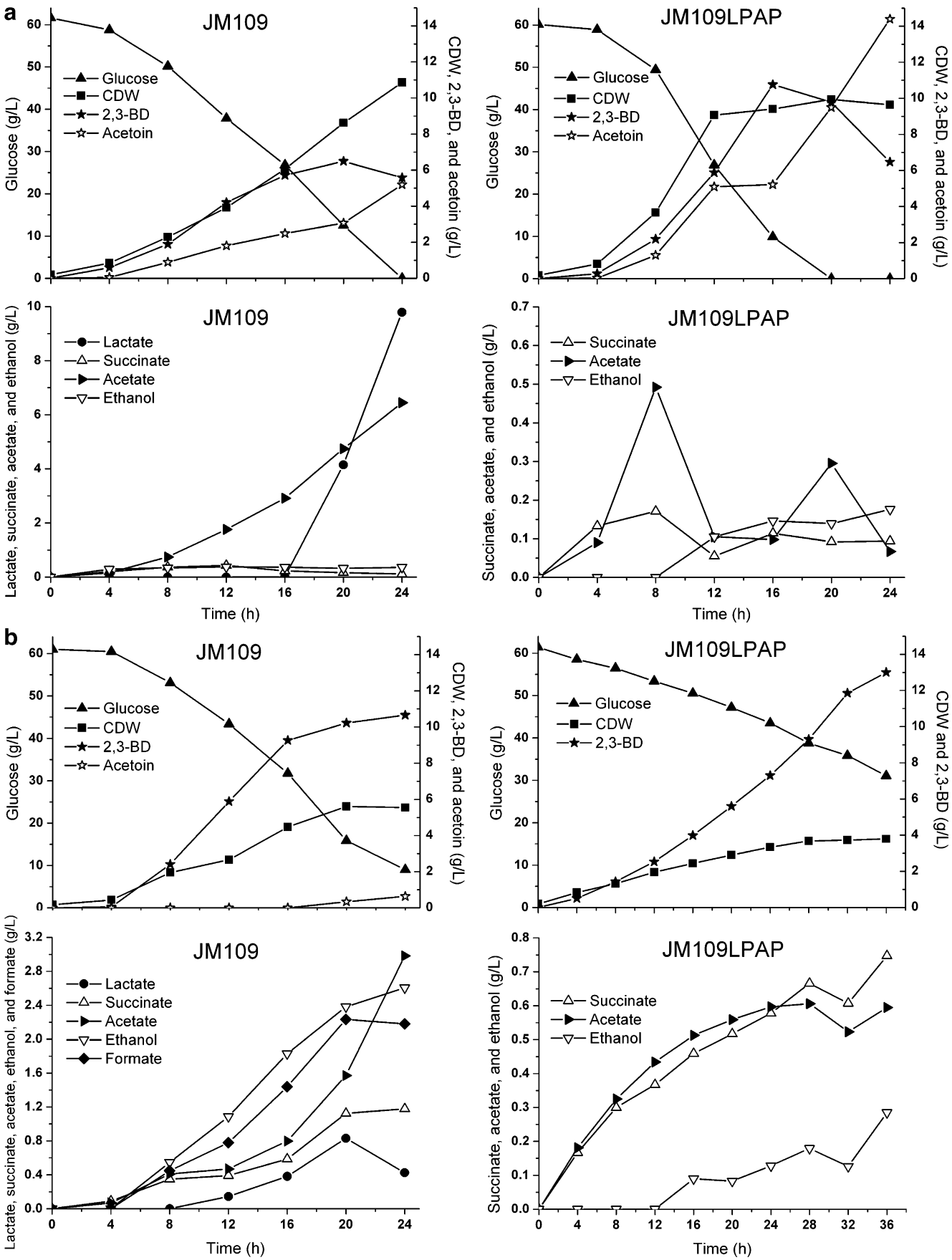
eliminated or decreased in the broth of the mutant, yet a slight increase of succinate formation was observed. The substrate to 2,3-BD and acetoin yield $Y_{BD+acetoin/glucose}$ was similar in both strains cultivated under high-aeration or low-aeration conditions (Table 4).

## Production of meso-2,3-BD under low oxygen condition in a 6-L fermentor

*E. coli* JM109 (pEnBD) and JM109LPAP (pEnBD) were cultivated in a 6-L fermentor for a batch process. Two dissolved oxygen concentrations were employed to maintain the different microaerobic conditions. When DO was set at 3% of air saturation, the mutant harboring pEnBD showed faster cell growth and glucose uptake rate compared to the control did, yet final cell dry weight was similar for both strains. When glucose concentration was lower than 10 g/L, a conversion of 2,3-BD to acetoin was observed. For *E. coli* JM109LPAP (pEnBD), the concentration of 2,3-BD reached the highest value 10.8 g/L at 16 h, while the parent strain only produced maximal 6.5 g/L 2,3-BD at 20 h. At the end of fermentation, the parent strain produced 9.8 g/L lactate and 6.4 g/L acetate accompanied with small amounts of succinate and ethanol. In contrast, only small amounts of succinate, acetate, and ethanol (lower than 0.2 g/L) were produced in the mixed acid fermentation pathway deleted mutant. The substrate to 2,3-BD and acetoin yield $Y_{BD+acetoin/glucose}$ was 0.35 g BD +acetoin/g glucose for *E. coli* JM109LPAP (pEnBD), about twice of 0.17 g BD+acetoin/g glucose of that produced by the parent strain.

When oxygen supply was further reduced to 1% DO of air saturation, the mutant harboring pEnBD exhibited slower cell growth and glucose consumption rate compared to that of the control, thus the final cell dry weight was much lower than that of *E. coli* JM109 (pEnBD). The mutant produced 13 g/L 2,3-BD without any acetoin after 30 g/L glucose was consumed in 36 h, whereas the parent strain consumed 52 g/L glucose and produced 11 g/L 2,3-BD and 0.6 g/L acetoin in a 24 h cultivation. As to the by-product formation, the parent strain produced a mixture of lactate, succinate, acetate, ethanol, and formate in significant amounts. However, only small amounts of succinate, acetate, and ethanol could be detected in the mixed acid fermentation pathway deleted mutant. In terms of substrate to 2,3-BD yield $Y_{BD/glucose}$, the mutant was 0.43 g BD/g glucose, significantly higher than 0.20 g BD/g glucose of the parent control.

**Fig. 3** Cell dry weight, 2,3-BD production, glucose consumption, and extracellular metabolite production profile of *E. coli* JM109(pEnBD) and JM109LPAP(pEnBD) grown in a 6-L fermentor under oxygen limited condition. **a** DO was controlled at 3%; **b** DO was controlled at 1%


a
JM109
Glucose
CDW
2,3-BD
Acetoin
Glucose (g/L)
CDW, 2,3-BD, and acetoin (g/L)
JM109LPAP
Glucose
CDW
2,3-BD
Acetoin
Glucose (g/L)
CDW, 2,3-BD, and acetoin (g/L)
JM109
Lactate
Succinate
Acetate
Ethanol
Lactate, succinate, acetate, and ethanol (g/L)
Time (h)
JM109LPAP
Succinate
Acetate
Ethanol
Succinate, acetate, and ethanol (g/L)
Time (h)
b
JM109
Glucose
CDW
2,3-BD
Acetoin
Glucose (g/L)
CDW, 2,3-BD, and acetoin (g/L)
JM109LPAP
Glucose
CDW
2,3-BD
Glucose (g/L)
CDW and 2,3-BD (g/L)
JM109
Lactate
Succinate
Acetate
Ethanol
Formate
Lactate, succinate, acetate, ethanol, and formate (g/L)
Time (h)
JM109LPAP
Succinate
Acetate
Ethanol
Succinate, acetate, and ethanol (g/L)
Time (h)

## Discussion

Heterologous expression of 2,3-BD producing operon with stereospecific enzymes in *E. coli* is an elegant method to obtain enantiomerically pure 2,3-BD isomers. Previous studies showed that effective 2,3-BD production could be achieved by recombinant *E. coli* harboring 2,3-BD producing genes under aerobic condition (Ui et al. 1997b; Ui et al. 2004; Yan et al. 2009). However, 2,3-BD is a typical substance of fermentation and the anaerobic or microaerobic producing processes are economically more feasible for reasons of simple reactor designs, easy control strategies, and convenient operation conditions. Thus we studied the possibility of 2,3-BD production under low oxygen condition in recombinant *E. coli*.

*E. coli* is a facultative anaerobe which can obtain energy by two different pathways: respiration and fermentation. When the oxygen supply is limited, mixed acid fermentation pathways are employed for glucose metabolism, producing ethanol, succinate, lactate, acetate, formate, hydrogen, and carbon dioxide as end-products. To obtain high 2,3-BD yield and productivity under low oxygen condition, genes including *ldhA*, *pta*, *adhE*, and *poxB* were deleted to alter the mixed acid fermentation pathways in *E. coli* JM109. Shake flask experiments under low-aeration condition were performed to evaluate the effects of gene deletions on glucose metabolism. Previous studies showed that the *ldhA* knockout in *E. coli* BW25113 caused a slight decrease for cell growth rate and glucose consumption under anaerobic conditions (Kabir et al. 2005). However, our studies demonstrated that the deletion of *ldhA* increased glucose uptake and cell growth in low-aeration shake flask cultivations (Table 3). Reasons could be regarded as the result of different oxygen utilization conditions, leading to modulation of cell metabolism to adapt for different intracellular oxidation states. With *ldhA* deletion, lactate production was totally blocked, while the production of acetate, succinate, and ethanol was promoted, indicating a shift of carbon flux for NAD regeneration, which was necessary to drive glycolysis and citric acid cycle. The ethanol production could not be totally eliminated by *adhE* knockout, probably due to the existence of alcohol dehydrogenase isoenzymes (Table 3). The knockout of *pta* in *ldhA* mutant slightly reduced acetate production, further deletion of *poxB* clearly decreased acetate formation (Table 3). In *E. coli*, acetate was mainly produced via two pathways: one is catalyzed by Pta-Ack and another by PoxB (Fig. 1). The simultaneous deletion of *pta* and *poxB* remarkably reduced acetate production, yet small amount of acetate was still present, which are attributed to the existences of isoenzymes catalyzing acetate formation from acetyl-CoA, e.g., the CoA transferase. The deletion of *poxB* revealed a significant reduction on glucose uptake, which agreed well with previous studies using *E. coli* BW25113. The carbon flux through glycolysis was reported to be reduced by *poxB* inactivation because of the down-regulation of enzyme activities involved in glycolysis and citric acid cycle, thus a reduction on glucose uptake and cell growth was observed (Li et al. 2007). When lactate, acetate, and ethanol producing pathways were eliminated or weakened, more carbon flux were diverted into succinate production to reduce the excess NADH to NAD, thus strain *E. coli* JM109LPAP produced the most succinate (Fig. 1 and Table 3).

When 2,3-BD producing pathway was introduced into *E. coli* strains via plasmid pEnBD, the *ldhA*, *pta*, *adhE*, and *poxB* quadruple deletion mutant exhibited faster glucose uptake and cell growth rate accompanied with higher 2,3-BD productivity under aerobic or limited oxygen conditions (Table 4 and Fig. 3a). However, when oxygen supply was lowered to 1% DO, the mutant grew much slower than the parent strain did (Fig. 3b). When the oxygen supply was met the demand of the cell respiration, the deletion of *ldhA* and *poxB* could block the conversion of pyruvate to lactate and acetate, thus more pyruvate can be used for 2,3-BD synthesis. Meanwhile, *adhE* and *pta* knockout allowed more acetyl-CoA to enter the citric acid cycle for energy supply (Fig. 1). Moreover, the reduced accumulation of by-products reduced the toxicity that retarded the cell growth. Considering the 2,3-BD fermentation pathway involves a cofactor balance process, thus, the mixed acid fermentation pathway deleted mutant showed faster cell growth and higher glucose uptake rate, leading to a favorable 2,3-BD production process (Table 4 and Fig. 3a). If the oxygen availability was further reduced, the respiration was limited further and cell began to implement fermentation for glucose metabolism and energy supply. For *E. coli* JM109LPAP (pEnBD), the lactate, acetate, and ethanol producing pathways were all blocked or weakened, which reduced the outlets of glucose metabolism and affected the regeneration of NAD, thus, defected cell growth and glucose uptake were observed (Fig. 3b). In fermentor studies, the wild type harboring 2,3-BD producing pathway excreted large amounts of lactate and acetate when oxygen was controlled at 3% DO. If the oxygen supply was further limited to 1% DO, the end-products of fermentation comprised a mixture of acetate, ethanol, formate, succinate, and lactate in significant amounts (Fig. 3a, b). These results indicated that the block of mixed acid fermentation pathway is important for effective 2,3-BD production under low oxygen condition, which could enhance 2,3-BD yield significantly.

When glucose concentration was at low level, a rapid transformation of 2,3-BD to acetoin was observed (Fig. 3a). However, if oxygen supply was limited to 1% DO, 2,3-BD became a major end-product of fermentation and the

formation of acetoin was clearly decreased or completely eliminated (Fig. 3b). Similar phenomena were reported previously (Ui et al. 1997b). These results indicated that a reduced intracellular state favored the conversion of acetoin to 2,3-BD, probably due to the catalysis property of acetoin reducase. Decreasing the oxygen supply rate would accelerate the conversion of acetoin to 2,3-BD, thus increase the 2,3-BD yield. However, the overall conversion rate was decreased due to a lower cell density.

In conclusion, the present study reveals the potential of enantiomerically pure 2,3-BD isomer production by recombinant *E. coli* under low oxygen condition. Under a limited oxygen supply, the disruption of mixed acid fermentation pathway improved cell growth and glucose uptake rate, thus enhanced 2,3-BD production. Further decrease on oxygen supply led to a major accumulation of 2,3-BD, yet decreased cell growth and 2,3-BD conversion rate. A further optimized control of oxygen supply will help to achieve a balance between 2,3-BD productivity and yield.

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