Appl Microbiol Biotechnol (2012) 95:461–469
DOI 10.1007/s00253-012-3883-9

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Deletion of lactate dehydrogenase in *Enterobacter aerogenes* to enhance 2,3-butanediol production

Moo-Young Jung · Chiam Yu Ng · Hyohak Song · Jinwon Lee · Min-Kyu Oh

Received: 31 October 2011 / Revised: 28 December 2011 / Accepted: 30 December 2011 / Published online: 3 February 2012

Abstract 2,3-Butanediol is an important bio-based chemical product, because it can be converted into several C4 industrial chemicals. In this study, a lactate dehydrogenase-deleted mutant was constructed to improve 2,3-butanediol productivity in *Enterobacter aerogenes*. To delete the gene encoding lactate dehydrogenase, λ Red recombination method was successfully adapted for *E. aerogenes*. The resulting strain produced a very small amount of lactate and 16.7% more 2,3-butanediol than that of the wild-type strain in batch fermentation. The mutant and its parental strain were then cultured with six different carbon sources, and the mutant showed higher carbon source consumption and microbial growth rates in all media. The 2,3-butanediol titer reached 69.5 g/l in 54 h during fed-batch fermentation with the mutant, which was 27.4% higher than that with the parental strain. With further optimization of the medium and aeration conditions, 118.05 g/l 2,3-butanediol was produced in 54 h during fed-batch fermentation with the mutant. This is by far the highest titer of 2,3-butanediol with *E. aerogenes* achieved by metabolic pathway engineering.

M.-Y. Jung · C. Y. Ng · M.-K. Oh (✉)
Department of Chemical & Biological Engineering,
Korea University,
Seoul 136-701, Republic of Korea
e-mail: mkoh@korea.ac.kr

H. Song
Research and Development Center, GS Caltex Corporation,
Daejeon 305-380, Republic of Korea

J. Lee
Department of Chemical & Biomolecular Engineering,
Sogang University,
Seoul 121-742, Republic of Korea

Keywords 2,3-Butanediol · *Enterobacter aerogenes* · Lactate dehydrogenase · Fed-batch fermentation

Introduction

2,3-Butanediol produced by microbes has attracted great interest because of the diversity of potential uses in the chemical industry. 2,3-Butanediol is an important chemical intermediate, which can be converted to 2-butanone or butadiene by dehydration, or to a polyester by esterification. The chemicals produced can be used to manufacture synthetic rubber, antifreeze agents, plastics, flavoring agents, and fuel additives (Celinska and Grajek 2009). 2,3-Butanediol production by *Klebsiella oxytoca* (Ji et al. 2010), *K. pneumonia* (Ma et al. 2009), *Paenibacillus polymyxa* (Gao et al. 2010), *Bacillus amyloliquefaciens* (Yang et al. 2011), *Serratia marcescens* (Zhang et al. 2010), and other strains has been recently reported. *Enterobacter aerogenes* was studied as a host for 2,3-butanediol production until the early 2000s. For example, the effects of pH, acetic acid, and oxygen level on 2,3-butanediol production with continuous culture have been studied in *E. aerogenes* (Zeng et al. 1990a, b). A membrane reactor for cell recycling was also developed and yielded 110 g/l 2,3-butanediol and acetoin with stepwise pH adaption at high cell density under fed-batch fermentation (Zeng et al. 1991). Perego et al. (2000) used several inexpensive carbon sources, such as starch hydrolysate, molasses, and whey and optimized culture conditions (temperature, pH, and initial glucose concentration) for 2,3-butanediol production. Converti and Perego (2002) conducted metabolic flux analyses with various initial glucose concentration conditions. Geckil et al. (2004) tried to increase 2,3-butanediol production by introducing the hemoglobin gene (*vgb*) of *Vitreoscilla*.

Reports of 2,3-butanediol production by *E. aerogenes* have mainly focused on the optimization of culture conditions and not on the metabolic pathway engineering.

A few studies have applied metabolic engineering to *E. aerogenes*. Zhao et al. (2009a, 2009b) demonstrated the effects of deleting formate hydrogen lyase subunit, deleting a hydrogenase subunit and overexpressing foreign formate dehydrogenase and its activator on hydrogen production in *E. aerogenes*. Lu et al. (2010) investigated the effect of deleting lactate dehydrogenase and overexpressing formate hydrogenase on hydrogen production. However, *E. aerogenes* metabolic engineering focusing on 2,3-butanediol production has not been intensively investigated yet.

E. aerogenes produces significant amounts of 2,3-butanediol from a wide range of carbon sources, while generating a number of soluble and gaseous products. Lactate is largely produced as a main by-product during *E. aerogenes* fermentation. Reducing lactate production can be beneficial to 2,3-butanediol production, because these two products compete with each other for pyruvate as a metabolic intermediate and NADH as a cofactor. In this study, we attempted to reduce lactate production by deleting the gene encoding lactate dehydrogenase from *E. aerogenes* (Fig. 1). The λ Red recombination method was adapted to *E. aerogenes* for this purpose. As expected, the deletion mutant showed much higher 2,3-butanediol production levels under both batch and fed-batch fermentation. The results demonstrated that *E. aerogenes* is another promising industrial strain for production of 2,3-butanediol.

Materials and methods

Strain development

The wild-type strain, *E. aerogenes* KCTC 2190, was obtained from the Korean Collection for Type Culture (KCTC). A lactate dehydrogenase (*ldhA*)-deleted mutant, EMY-01, was developed from *E. aerogenes* KCTC 2190 using the λ Red recombination method (Datsenko and Wanner 2000). Briefly, the *E. aerogenes* KCTC 2190 genome sequence was kindly provided by Macrogen Inc. (Seoul, South Korea). The *Escherichia coli ldhA* homolog was identified using BLAST and used to design *ldhA*-Kan-fw and *ldhA*-Kan-rv (Table 1) to amplify the 1.79-kb PCR product of the kanamycin resistance (*kan*) gene on a thermal cycler with pKD4 as the template (MJ MiNi, Bio-Rad, Hercules, CA, USA).

Then, the pKM208 plasmid was transformed into *E. aerogenes* KTCT 2190 grown in Luria Broth (LB) media using an electroporator at 2100 V and selected on LB plates containing 400 μg/ml carbenicilln at 30 °C. The pKM208 plasmid contained the *λ red* and *gam* genes under the *tac* promoter (Murphy and Campellone 2003). *E. aerogenes* containing pKM208 was cultured at 30 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM when OD_{600} reached 0.2. The linear polymerase chain reaction (PCR) product of the *kan* gene was transformed using electroporation when OD_{600} reached 0.6. The transformants were selected at 37 °C on LB agar plate containing 50 μg/ml kanamycin. Deletion of the *ldhA*

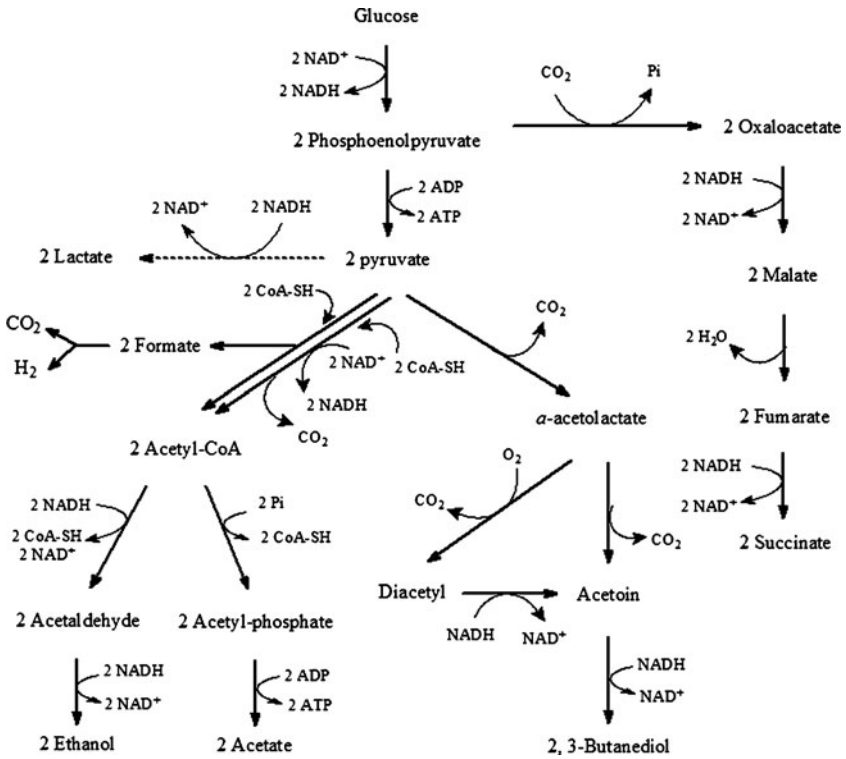

Fig. 1 Fermentation pathways in *Enterobacter aerogenes* (modified from Ji et al. 2011). The *dotted line* is the deleted pathway in this study

Table 1 Strains, plasmids, and primers used in this study

Strain, plasmids or primers	Genotype, relevant characteristics or sequence	Source or reference
Strains		
E. aerogenes KCTC 2190	Wild type	Korean Collection for Type Culture
EMY 01	*E.aerogenes* KCTC 2190 △*ldhA*	This work
Plasmids		
pKM208	*lac*I, λ *Red* + *Gam*-producing vector, tac_promoter, f1_ori, Amp^R	Addgene
pCP20	FLP recombinase producing vector, Amp^R, Cm^R	Datsenko and Wanner (2000)
pKD4	FRT flanked resistance cassette involved vector, oriRγ, Km^R	Datsenko and Wanner (2000)
Primers		
ldhA-Kan-fw	ATTATTTTAATTATGCTACCGTGACGGTATTATCACT AGAGGAAAGCCTTGTGTAGGCTGGAGCTGCTTC	This study
ldhA-Kan-rv	TTGTCTGAATGTGCCCCCCGTTAGCGGGGAGCACA AAAGGGGAAAGAAGCTCCTCCTTAGTTCCTATTCC	This study
ldhA-Con-A	GTATTATCACTAGAGGAAAGCCTT	This study
ldhA-Con-B	CATATTCAACGCCGAGATCC	This study
ldhA-Con-C	CGGTGCTGGAAGAGCTAAAA	This study
ldhA-Con-D	GCACAAAAGGGGAAAGAAGC	This study
ldhA-Con-Bk	CTCGTCCTGCAGTTCATTCA	This study
ldhA-Con-Ck	GGATGATCTGGACGAAGAGC	This study

Underlined sequences were taken from the *ldhA* homolog of *Enterobacter aerogenes* KCTC 2190

homolog was confirmed by PCR using the *ldhA*-Con-A/*ldhA*-Con-Bk and *ldhA*-Con-Ck/*ldhA*-Con-D primer sets (Table 1). The loss of pKM208 from the strain was confirmed by a carbenicillin sensitive colony on an LB agar plate. Then, the pCP20 plasmid was transformed into the *ldh* knockout mutant of *E. aerogenes* using electroporation to remove the *kan* gene by FRT recombination. The colony grown on the LB plate containing 50 μg/ml chloramphenicol at 30 °C was cultured in 1 ml LB medium at 42 °C overnight, and then a kanamycin-sensitive, chloramphenicol-sensitive colony was selected on the LB plate. The strains, plasmids, and primers used in this study are listed in Table 1.

Media and culture conditions

The medium (1 l) to produce 2,3-butanediol was modified from Petrov and Petrova (2009) and consisted of 3 g KH_2PO_4, 6.8 g Na_2HPO_4, 0.75 g KCl, 5.35 g $(NH_4)_2SO_4$, 0.28 g Na_2SO_4, 0.26 g $MgSO_4{\cdot}7H_2O$, 0.42 g citric acid, 5 g yeast extract, and 0.3 ml microelement solution (1 l) containing 34.2 g $ZnCl_2$, 2.7 g $FeCl_3$ $6H_2O$, 10 g $MnCl_2$ $4H_2O$, 0.85 g $CuCl_2$ $2H_2O$, and 0.31 g H_3BO_3. This medium was used for all flask and fermentation processes. In flask culture, different carbon sources were additionally added to the medium as indicated in the results. *E. aerogenes* KCTC 2190 and EMY-01 were cultured micro-aerobically in a 250-ml flask containing 50 ml medium at 37 °C with shaking at 250 rpm.

The batch and fed-batch fermentations were conducted in a 5-l stirring bioreactor (Bio Control and System, Daejeon, Korea) with a working volume of 3 l with 5% (v/v) inoculum. For batch fermentation, initial glucose concentration in the medium was 90 g/l, and temperature, pH, and agitation speed were maintained at 37 °C, 6.0, and 150 rpm, respectively. pH was adjusted with 5 M NaOH, and antifoam 204 (Sigma, St. Louis, MO, USA) was added whenever needed. The aerobic environment in the bioreactor was maintained by aerating air at 1 vvm. Fed-batch fermentation was performed by adding 60 g/l glucose into the initial medium, and the feeding solution consisted of 700 g/l glucose and 20 g/l $MgSO_4$ $7H_2O$. The glucose level was maintained at <40 g/l. Other conditions were the same as those for the batch fermentation unless indicated in "Results".

Analytical methods

The optical density of the bacteria was measured at 600 nm (OD_{600}) using a UV–Vis spectrophotometer (Shimazu UV mini 1240, Tokyo, Japan). One milliliter of fermentation media was transferred to a microcentrifuge tube to measure concentration. After centrifugation for 5 min at 13,000 rpm, the supernatant was transferred to a new tube and used for the metabolite assay. 2,3-Butanediol, acetoin, lactate, succinate, formate, acetate, ethanol, glucose, glycerol, galactose, fructose, sucrose, and xylose were analyzed by high-performance

liquid chromatography (Younglin Instrument ACME-9000, Seoul, South Korea) equipped with a Sugar SH1011 column (Shodex, Tokyo, Japan) and an RI-detector at 45 °C. The column heater was set at 75 °C, and 0.01 M sulfuric acid was used as the mobile phase at a flow rate of 0.5 ml/min. Glucose concentration was assayed with the RQflex plus 10 (Merck, Darmstadt, Germany) during fed-batch fermentation.

Results

Construction of the *E. aerogenes* KCTC 2190*ldh*-deleted mutant

Lactate is a major by-product of *E. aerogenes* KCTC 2190 during 2,3-butanediol production (Lu et al. 2010). For deletion of the gene encoding lactate dehydrogenase, the genome sequence of *E. aerogenes* KCTC 2190 was kindly provided by Macrogen, Inc. An open reading frame of which the protein sequence has 90% sequence identity with *E. coli* lactate dehydrogenase A was successfully identified. For developing gene deletion method (Fig. 2a), antibiotics resistance of *E. aerogenes* was evaluated. The strain was sensitive to 50 μg/ml kanamycin and chloramphenicol but was resistant to 500 μg/ml ampicillin. However, it was unable to grow on media with 400 μg/ml carbenicillin, which is a more stable antibiotic than ampicillin but degradable with β-lactamase. Therefore, the *ldhA* deletion mutant was developed with a kanamycin resistance gene marker in pKD4, and transformants containing pKM208 and pCP20 were selected by carbenicillin and chloramphenicol, respectively. The deleted mutation with the *ldhA::kan* gene was reconfirmed by colony PCR using the primers *ldhA*-Con-A, *ldhA*-Con-B, *ldhA*-Con-C, *ldhA*-Con-D, *ldhA*-Con-Bk, and *ldhA*-Con-Ck (Fig. 2b). Because *ldhA*-Con-B and *ldhA*-Con-C have wild-type *E. aerogenes ldhA* gene sequence and the *ldhA*-Con-Bk and *ldhA*-Con-Ck contain the sequence for the kanamycin resistance gene (*kan*), the deletion mutants were confirmed by PCR with those primer sets (Fig. 2c). After the deleted *ldhA::kan* mutation was successfully obtained, the kanamycin resistance gene was removed by FRT recombination with FLP by transforming pCP20. After selecting kanamycin-sensitive and chloramphenicol-sensitive colonies, the □*ldhA* gene was confirmed by the reduced size (144 bp) of the PCR products using the primers *ldhA*-Con-A and *ldhA*-Con-D (data not shown). One of the colonies was named EMY-01.

Effects of *ldhA* deletion on batch fermentation

Batch fermentation of *E. aerogenes* KCTC 2190 and EMY-01 was performed at a 90-g/l initial glucose concentration. As shown in Table 2, disruption of *ldhA* resulted in a 97% decrease in lactate production, confirming that the major lactate dehydrogenase producing gene in *E. aerogenes*

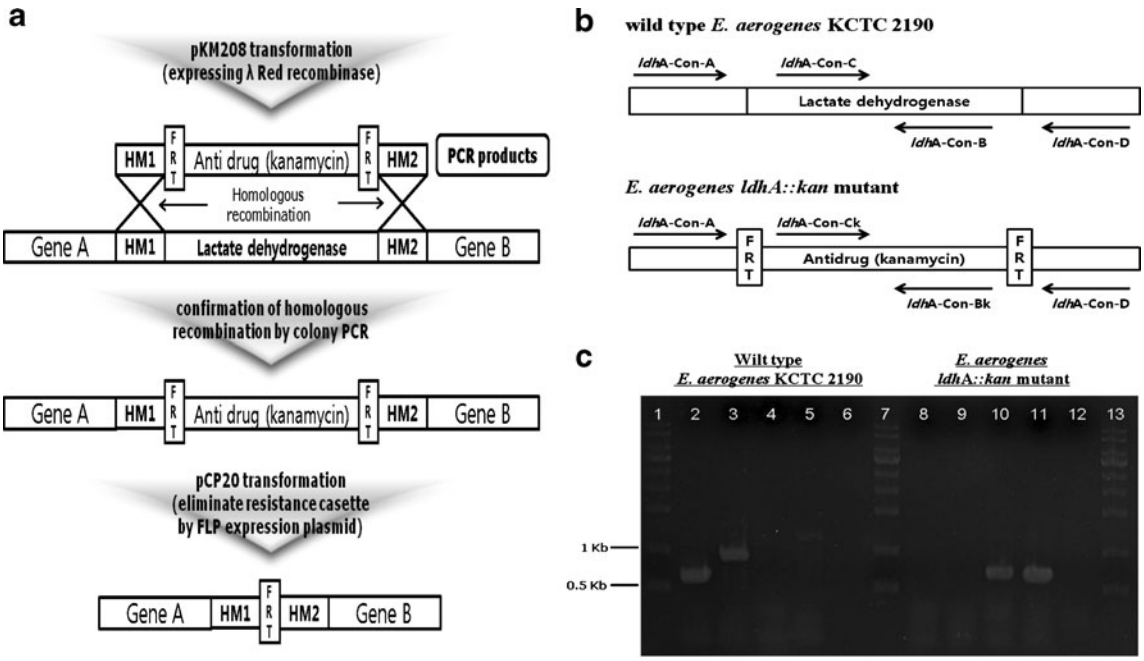

Fig. 2 Deletion of a lactate dehydrogenase gene in the genomic DNA. **a** Scheme for *ldhA* deletion using the λ Red recombination method. **b** Primer binding region for polymerase chain reaction (PCR) confirmation of successful homologous recombination. The wild-type *Enterobacter aerogenes* genomic DNA produced PCR products using the *ldhA*-Con-A/-B and *ldhA*-Con-C/-D primer sets, whereas *ldhA* mutant did so with the *ldhA*-Con-A/-Bk and *ldhA*-Con-Ck/-D primers. **c** Agarose gel electrophoresis of colony PCR products. *Lanes 1*, *7*, and *13*: 1-kb size marker; *lanes 2* and *8*: PCR with *ldhA*-Con-A/-B primers; *lanes 3* and *9*: *ldhA*-Con-C/-D; *lanes 4* and *10*: *ldhA*-Con-A/-Bk; *lanes 5* and *11*: *ldhA*-Con-Ck/-D; *lanes 6* and *12*: blank

Table 2 Comparison of final concentrations of metabolites, glucose consumption, 2,3-butanediol productivity, and yield obtained between wild-type *Enterobacter aerogenes* and the *ldhA*-deleted mutant EMY-01 after 12 h of batch fermentation

Strains	*E. aerogenes* KCTC 2190	EMY-01
Consumption of glucose (g/l)	74.1	76.5
2,3-Butanediol (g/l)	19.88	23.2
Biomass (g/l)	5.43	6.85
Acetoin (g/l)	0.42	0.51
Lactate (g/l)	9.88	0.34
Succinate (g/l)	3.34	4.18
Formate (g/l)	0.40	0.81
Acetate (g/l)	0.30	0.24
Ethanol (g/l)	7.93	9.55
2,3-Butanediol productivity (g/l/h)	1.66	1.93
2,3-Butanediol yield (g/g Glc)	0.27	0.30

KCTC 2190 was successfully disrupted. A small amount of lactate (0.34 g/l) was produced by a minor lactate dehydrogenase or another dehydrogenase. The significant decrease in lactate production was beneficial for 2,3-butanediol productivity and yield, which increased 16.3% and 11.1%, respectively. The concentrations of other fermented products, such as succinate, formate, and ethanol, all increased >30% in the mutant strain. However, the absolute change in these byproducts was not as significant as that of 2,3-butanediol in the mutant. Interestingly, microbial biomass production and glucose consumption rate by the mutant increased by 26.2% and 3.2% respectively compared to those by the wild-type strain, which was qualitatively consistent with a previous report (Lu et al. 2010).

Carbon source effects on the *ldhA*-deleted mutant

E. aerogenes utilizes various carbon sources effectively (Perego et al. 2000). Flask cultivations were conducted with 40 g/l of six carbon sources such as glucose, galactose, fructose, sucrose, xylose, and glycerol to test the metabolic effects of the *ldhA* deletion (Table 3). With glucose, 2,3-butanediol, biomass production, and glucose consumption were more significantly increased by the *ldhA*-deleted strain in flask culture compared to those in batch fermentation. It was suggested that the reduced pH decrease in the *ldhA* mutant cultivation media resulted in the increase (Booth 1985). The wild-type strain must have undergone a significant pH shock in the flask culture without a pH adjustment resulting in a slower growth rate than that of the mutant, and 2,3-butanediol production was suppressed accordingly. However, the *ldhA* mutant underwent less of a pH shock than that of the wild-type, so more significant increases in biomass and 2,3-butanediol production were observed.

Carbon source consumption, biomass, and 2,3-butanediol production by the *ldhA* mutant increased similarly with galactose, fructose, and sucrose (Table 3). These carbon sources are consumed in the glycolytic pathway, similar to glucose. Interestingly, biomass production was the highest with fructose, but the reason was unclear. The wild-type *E. aerogenes* was ineffective for converting xylose to 2,3-butanediol due to its low xylose consumption rate. This problem was partly overcome with the *ldhA* mutant, which consumed xylose much faster. Although 2,3-butanediol production with added xylose was about 30% lower than that with glucose, this result strongly suggested that the *ldhA* mutant would be a much better strain when lignocellulosic biomass is used as the carbon source. The glycerol consumption rate also increased dramatically after the *ldhA* mutation. Interestingly, the increased glycerol consumption resulted in only a small increase in 2,3-butanediol production but a significant increase in ethanol production. Overall, the *ldhA* mutation enhanced biomass and 2,3-butnaediol production with all six carbon sources, but the increase in 2,3-butanediol production was not dramatic with the use of glycerol.

Improvement of 2,3-butanediol production by fed-batch fermentation

Microbial biomass, 2,3-butanediol, and major byproducts of wild-type *E. aerogenes* and EMY-01 were monitored in fed-batch cultures. As shown in Fig. 3, 2,3-butanediol production by EMY-01 reached 69.12 g/l within 54 h of cultivation, which was 28.5% higher than that by its parent strain. Additionally, the mutant produced 0.35 g/l lactate, which was a 98.3% reduction when compared to that of the wild-type strain. However, an improvement of microbial growth rate in batch culture was not observed in fed-batch fermentation. Rather, the reduced death rate of *ldhA* mutant provided a beneficial effect on 2,3-butanediol production. Among the by-products, the mutant strain produced 18.87 g/l succinate and 12.31 g/l ethanol, which were increases of 67.4% and 53.7% compared to those in the wild-type strain. Succinate production was higher than that of ethanol in the fed-batch culture.

The lactate dehydrogenase deletion mutant was obviously a better strain for producing 2,3-butanediol in both batch and fed-batch cultures. Nevertheless, the lower biomass production in fed-batch culture remained an issue to be resolved. We hypothesized that the low biomass production was caused by an amino acid limitation in the media. Hence, casamino acid was added to the medium (10 g/l). Additionally, the agitation rate and aeration were increased from 150 to 280 rpm and from 1 to 2 vvm, respectively, to improve respiration rate of the strain. A biomass concentration of 10.52 g/l was obtained within 34 h of cultivation, which was an increase of 124.8% compared to that of the previous

Table 3 Comparison of *Enterobacter aerogenes* KCTC 2190 and the *ldhA*-deleted mutant EMY-01 using several carbon sources in a 12-h flask cultivation

Strains	*E. aerogenes* KCTC 2190						EMY-01					
Carbon source (40 g/l)	Glucose	Galactose	Fructose	Sucrose	Xylose	Glycerol	Glucose	Galactose	Fructose	Sucrose	Xylose	Glycerol
Consumed carbon source (g/l)	33.97	26.73	29.76	30.66	22.40	18.50	39.89	37.20	37.68	39.24	28.74	27.90
2,3-Butanediol (g/l)	9.49	8.73	9.65	9.28	6.34	0.23	12.52	12.21	12.70	12.17	8.61	1.12
Biomass (g/l)	3.05	3.04	4.67	3.55	2.81	1.81	3.45	4.42	4.73	4.4	4.09	2.82
Acetoin (g/l)	0.55	0.75	0.65	0.64	0.67	0.07	1.21	0.95	0.90	0.57	0.91	0.15
Lactate (g/l)	1.33	0.82	1.61	0.68	0.54	1.96	0.06	0.00	0.00	0.00	0.00	0.33
Ethanol (g/l)	5.01	3.99	4.34	5.17	3.69	8.15	5.71	6.13	5.87	7.01	4.44	12.83
Succinate (g/l)	1.54	1.83	2.46	2.09	1.81	0.83	1.92	2.26	2.74	2.49	2.17	1.41
2,3-Butanediol productivity (g/l/h)	0.79	0.73	0.80	0.77	0.53	0.02	1.04	1.02	1.06	1.01	0.72	0.10
2,3-Butanediol yield (g/g Glc)	0.28	0.33	0.32	0.30	0.28	0.01	0.31	0.33	0.34	0.31	0.30	0.04

The data are averages of two independent experiments

fermentation (Fig. 3c). Consequently, a 2,3-butanediol concentration of 108.8 g/l with a yield of 0.42 was achieved at 34 h. The productivity was 3.2 g/(l h) until this time, which was a 130% increase when compared to the previous 54 h of fermentation. Productivity decreased noticeably after 34 h, and production almost ceased at 54 h. At the end of the fermentation (54 h), 118.05 g/l of 2,3-butanediol was obtained, and this was the highest titer ever reported for *E. aerogenes*.

Discussion

We investigated deletion of the lactate dehydrogenase gene to improve 2,3-butanediol production in *E. aerogenes* KCTC 2190 for two reasons. First, lactate is the most abundant by-product that consumes carbon flux from glucose in *E. aerogenes*. Second, 1 mol of NADH is consumed when 1 mol of lactate is produced, which is needed to produce 2,3-butanediol. Therefore, the effect of lactate dehydrogenase on 2,3-butanediol production was examined using the deletion mutant. Three candidate genes encoding lactate dehydrogenase were found by the BLAST program in the *E. aerogenes* genome sequence. Among them, one with 90% protein sequence identity to lactate dehydrogenase A in *E. coli* was chosen as the target.

Because both *E. aerogenes* and *E. coli* are Gram-negative members of the Enterobacteriaceae family, we tried to apply λ Red recombination as the deletion method, which worked with *E. coli*. The first few trials were unsuccessful, but deletion mutants were successfully acquired after substituting the pKD46 with pKM208 λ Red expression vectors. The difference in these two vectors was the promoter for expressing *exo*, *beta*, and *lambda*, which are involved in efficient homologous recombination. Possibly, P_{BAD} in pKD46 was not active at the start of transcription, or arabinose could not properly induce the promoter in *E. aerogenes*. This problem was solved by using P_{tac} of pKM208 and IPTG as an inducer. We successfully constructed the *ldhA* mutant using this method, and lactate production decreased >97% in the mutant.

Deletion of lactate dehydrogenase is beneficial for 2,3-butanediol production because of the re-direction in carbon flux and increased NADH availability. Another interesting benefit of the deletion was an increase in microbial cell growth. Generally, a metabolically engineered microbe has a reduced growth rate compared to its parental strain. The main reason for the increased microbial growth of the mutant must be that reducing lactate production lowered the acidification rate of the media. Indeed, batch fermentation with the *ldhA* mutant consumed 11.8 ml of 5 M NaOH to maintain the pH at 6.0 for 12 h of cultivation, which was only 11% of the amount used by the wild-type fermentation. A high amount of NaOH in the culture media causes osmotic stress to microbial cells as well as cell lysis (Petrov and Petrova 2010). An increase of microbial biomass production was not observed in fed-batch fermentation, but the microbial death rate was clearly reduced by the *ldhA* deletion (Fig. 3a, b). This could also be caused by the reduced amount of NaOH used in the fed-batch fermentation with the *ldhA* mutant.

The effect of the lactate dehydrogenase deletion was tested using six different carbon sources. The *ldhA* mutant showed improvement in carbon source consumption rates, biomass, and 2,3-butanediol production with all carbon sources. An interesting result was that when glycerol was

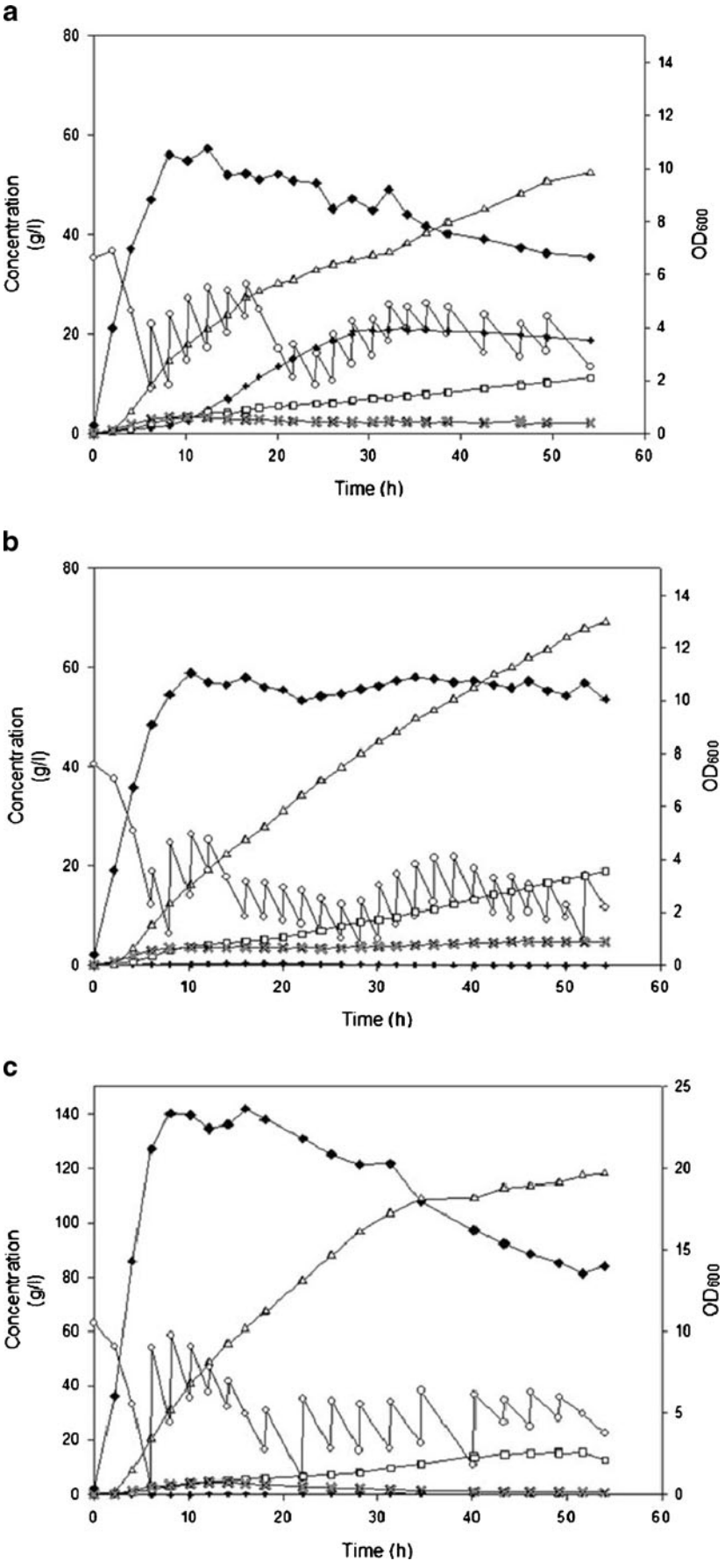

Fig. 3 Results of the fed-batch fermentation. **a** OD_{600} and metabolite concentrations of *Enterobacter aerogenes* KCTC 2190. **b** OD_{600} and metabolite concentrations of *E .aerogenes* EMY-01. **c** OD_{600} and metabolite concentrations of *E. aerogenes* EMY-01 after adding 10 g/l casamino acid and increasing aeration. *Symbols* represent optical density (*black diamond*), 2,3-butanediol (*white triangle*), glucose (*white circle*), lactate (*plus*), succinate (*white square*), and ethanol (*cross*)

used as the major carbon source, a significant increase in ethanol rather than 2,3-butanediol production was observed in the *ldhA* mutant. Indeed, in *E. coli*, ethanol (86%) and succinate (7%) are the major products when glycerol is used as a major carbon source, rather than acetate as the main metabolite when glucose is used as the carbon source (Dharmadi et al. 2006). Glycerol is a more reduced substrate than glucose, and therefore, two more extra hydrogens should be consumed during the fermentation. The *ldhA* mutant is likely to use ethanol production pathway to regenerate NAD + under oxygen-limited conditions.

The importance of deleting lactate dehydrogenase was demonstrated in our batch and fed-batch fermentation of 2,3-butanediol production. However, the low *E. aerogenes* density in fed-batch fermentation was a hurdle to improve 2,3-butanediol productivity. We tried to resolve this in two ways. We added amino acids to the medium and enhanced oxygen supply. Amino acid limitations are one of the most common problems during fermentation, which can be overcome by adding casamino acid. Additionally, more aeration possibly increased respiration rate, resulting in increased biomass production. The combination of these changes in the fed-batch fermentation successfully enhanced microbial biomass production and 2,3-butanediol productivity more than twofold (Fig. 3c). In particular, adding casamino acid resulted in a significant improvement in microbial growth in the flask culture experiment (data not shown). To search for the limiting amino acid, we added 20 amino acids individually to the flask culture media, but none of them resulted in a positive microbial growth effect. It is suspected that a few amino acids or a certain amino acid pathway was limited, which is currently under investigation in our laboratory. Finding factors limiting microbial growth must be a way to develop a more economical and efficient bioprocess for 2,3-butanediol production.

In summary, deleting the lactate dehydrogenase gene in *E. aerogenes* improved 2,3-butanediol production. An efficient *E. coli* gene disruption method was successfully modified and applied to *E. aerogenes* to develop the engineered strain. The *ldhA* deletion mutant provided much higher 2,3-butanediol production than its parental strain in both batch and fed-batch fermentations. Enhanced 2,3-butanediol production was achieved by supplying casamino acid and more oxygen to the medium, which resulted in 118 g/l 2,3-butanediol in 54 h. This amount was superior to the result of a previous *E. aerogenes* study conducted to optimize bioprocess conditions (Zeng et al. 1991). This study demonstrated the importance of metabolic pathway engineering in the process development for bio-based chemical production.

Acknowledgements This research was supported by the R&D Program of MKE/KEIT (No. 10035578, Development of 2,3-butanediol and derivative production technology for C-Zero bio-platform industry). The authors would also like to thank Macrogen, Inc. for providing the *E. aerogenes* KCTC2190 full genome sequence.

References

Booth IR (1985) Regulation of cytoplasmic pH in bacteria. Microbiol Rev 49:359–378

Celinska E, Grajek W (2009) Biotechnological production of 2,3-butanediol—current state and prospects. Biotechnol Adv 27:715–725

Converti A, Perego P (2002) Use of carbon and energy balances in the study of the anaerobic metabolism of *Enterobacter aerogenes* at variable starting glucose concentrations. Appl Microbiol Biotechnol 59:303–309

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

Dharmadi Y, Murarka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829

Gao JA, Xu H, Li QJ, Feng XH, Li S (2010) Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce R, R-2,3-butanediol. Bioresource Technol 101:7076–7082

Geckil H, Barak Z, Chipman DM, Erenler SO, Webster DA, Stark BC (2004) Enhanced production of acetoin and butanediol in recombinant *Enterobacter aerogenes* carrying *Vitreoscilla* hemoglobin gene. Bioprocess Biosyst Eng 26:325–330

Ji XJ, Huang H, Ouyang PK (2011) Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnol Adv 29:351–364

Ji XJ, Huang H, Zhu JG, Ren LJ, Nie ZK, Du J, Li S (2010) Engineering *Klebsiella oxytoca* for efficient 2,3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. Appl Microbiol Biotechnol 85:1751–1758

Lu Y, Zhao H, Zhang C, Lai Q, Wu X, Xing XH (2010) Alteration of hydrogen metabolism of *ldh*-deleted *Enterobacter aerogenes* by overexpression of NAD+ -dependent formate dehydrogenase. Appl Microbiol Biotechnol 86:255–262

Ma CQ, Wang AL, Qin JY, Li LX, Ai XL, Jiang TY, Tang HZ, Xu P (2009) Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Appl Microbiol Biotechnol 82:49–57

Murphy KC, Campellone KG (2003) Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. BMC Mol Biol 4:11

Perego P, Converti A, Del Borghi A, Canepa P (2000) 2, 3-Butanediol production by *Enterobacter aerogenes*: selection of the optimal conditions and application to food industry residues. Bioprocess Biosyst Eng 23:613–620

Petrov K, Petrova P (2009) High production of 2,3-butanediol from glycerol by *Klebsiella pneumoniae* G31. Appl Microbiol Biotechnol 84:659–665

Petrov K, Petrova P (2010) Enhanced production of 2,3-butanediol from glycerol by forced pH fluctuations. Appl Microbiol Biotechnol 87:943–949

Yang T, Rao Z, Zhang X, Lin Q, Xia H, Xu Z, Yang S (2011) Production of 2,3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. J Basic Microbiol. doi:10.1002/jobm.201100033

Zeng AP, Biebl H, Deckwer WD (1990a) 2, 3-Butanediol production by *Enterobacter aerogenes* in continuous culture: role of oxygen supply. Appl Microbiol Biotechnol 33:264–268

Zeng AP, Biebl H, Deckwer WD (1990b) Effect of pH and acetic acid on growth and 2,3-butanediol production of *Enterobacter aerogenes* in continuous culture. Appl Microbiol Biotechnol 33:485–489

Zeng AP, Biebl H, Deckwer WD (1991) Production of 2,3-butanediol in a membrane bioreactor with cell recycle. Appl Microbiol Biotechnol 34:463–468

Zhang LY, Sun JA, Hao YL, Zhu JW, Chu J, Wei DZ, Shen YL (2010) Microbial production of 2,3-butanediol by a surfactant (serrawettin)-deficient mutant of *Serratia marcescens* H30. J Ind Microbiol Biotechnol 37:857–862

Zhao HX, Ma K, Lu Y, Zhang C, Wang LY, Xing XH (2009a) Cloning and knockout of formate hydrogen lyase and H(2)-uptake hydrogenase genes in *Enterobacter aerogenes* for enhanced hydrogen production. Int J Hydrogen Energy 34:186–194

Zhao HX, Ma K, Lu Y, Zhang C, Wang LY, Xing XH (2009b) Perturbation of formate pathway for hydrogen production by expressions of formate hydrogen lyase and its transcriptional activator in wild *Enterobacter aerogenes* and its mutants. Int J Hydrogen Energy 34:5072–5079