High production of 2,3-butanediol from glycerol without 1,3-propanediol formation by *Raoultella ornithinolytica* B6

Taeyeon Kim[1,2] · Sukhyeong Cho[3] · Han Min Woo[4] · Sun-Mi Lee[1,5] · Jinwon Lee[3,6] ·
Youngsoon Um[1,5] · Jin-Ho Seo[2,7]

Abstract Conversion of crude glycerol derived from biodiesel processes to value-added chemicals has attracted much attention. Herein, *Raoultella ornithinolytica* B6 was investigated for the high production of 2,3-butanediol (2,3-BD) from glycerol without 1,3-propanediol (1,3-PD) formation, a byproduct hindering 2,3-BD purification. By evaluating the effects of temperature, agitation speed, and pH control strategy, the fermentation conditions favoring 2,3-BD production were found to be 25 °C, 400 rpm, and pH control with a lower limit of 5.5, respectively. Notably, significant pH fluctuations which positively affect 2,3-BD production were generated by simply controlling the lower pH limit at 5.5. In fed-batch fermentation under those conditions, *R. ornithinolytica* B6 produced 2,3-BD up to 79.25 g/L, and further enhancement of 2,3-BD production (89.45 g/L) was achieved by overexpressing homologous 2,3-BD synthesis genes (the *budABC*). When pretreated crude glycerol was used as a sole carbon source, *R. ornithinolytica* B6 overexpressing *budABC* produced 78.10 g/L of 2,3-BD with the yield of 0.42 g/g and the productivity of 0.62 g/L/h. The 2,3-BD titer, yield, and productivity values obtained in this study are the highest 2,3-BD production from glycerol among 1,3-PD synthesis-deficient 2,3-BD producers, demonstrating *R. ornithinolytica* B6 as a promising 2,3-BD producer from glycerol.

Keywords 2,3-Butanediol · *Raoultella ornithinolytica* · Fed-batch fermentation · *budABC* · Crude glycerol

Introduction

Microbial production of 2,3-butanediol (2,3-BD) from renewable resources is a promising alternative to conventional chemical processes. 2,3-BD is a value-added bio-based chemical with diverse applications in the food, pharmaceutical, and synthetic rubber industries as well as in transport fuel production (Celinska and Grajek 2009).

Sugar-based 2,3-BD fermentation has been extensively studied due to the high 2,3-BD production capabilities of various microorganisms including *Bacillus amyloliquefaciens*

✉ Youngsoon Um
  yum@kist.re.kr

✉ Jin-Ho Seo
  jhseo94@snu.ac.kr

1   Clean Energy Research Center, Korea Institute of Science and Technology (KIST), Seoul, Republic of Korea

2   Interdisciplinary Program in Agricultural Biotechnology, Collage of Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea

3   C1 Gas Refinery R&D Center, Sogang University, Seoul, Republic of Korea

4   Department of Food Science and Biotechnology, Sungkyunkwan University, Suwon, Republic of Korea

5   Clean Energy and Chemical Engineering, Korea University of Science and Technology, Daejeon, Republic of Korea

6   Department of Chemical and Biomolecular Engineering, Sogang University, Seoul, Republic of Korea

7   Department of Agricultural Biotechnology and Center for Food and Bioconvergence, Seoul National University, Seoul, Republic of Korea

(Yang et al. 2012), *Serratia marcescens* (Zhang et al. 2010), *Enterobacter aerugenes* (Jung et al. 2012), *Klebsiella pneumoniae* (Ma et al. 2009; Tsvetanova et al. 2014), and *Klebsiella oxytoca* (Cho et al. 2015b; Ji et al. 2009; Kim et al. 2013). Those previous sugar-based 2,3-BD titers have been enhanced over 100 g/L by optimizing the fermentation conditions (e.g., a two-agitation speed strategy and feeding strategy), medium compositions, and engineering microorganisms (e.g., deletion of lactic acid synthesis genes and over-expression of 2,3-BD synthesis-related genes).

Alternatively, 2,3-BD can be produced from glycerol, which is a major by-product from biodiesel-manufacturing processes. Biodiesel-derived glycerol (crude glycerol) is generated at a weight ratio of 1:10 (glycerol/biodiesel). Because biodiesel production has increased, use of crude glycerol as a carbon source for fermentation has attracted much attention for the production of chemicals such as ethanol, 1,3-propanediol (1,3-PD), citric acid, poly(hydroxyalkanoates), organic acids (lactic acid, succinic acid, propionic acid, etc.), and 2,3-BD (Clomburg and Gonzalez 2013; Feng et al. 2014; Luo et al. 2016). Among the natural 2,3-BD producers, *K. pneumoniae* (Petrov and Petrova 2009; Petrov and Petrova 2010) and *K. oxytoca* (Cho et al. 2015a) tend to produce 2,3-BD as the main product and 1,3-PD as a by-product from glycerol under aerobic conditions. Because glycerol is a more reduced carbon source than glucose, 1,3-PD formation involving net NADH consumption is usually observed in glycerol fermentation by *K. pneumoniae* and *K. oxytoca* in order to maintain a redox balance (i.e., NADH/NAD balance). 1,3-PD can be used as a monomer for polytrimethylene tere-phthalate used in the fiber and textile industries (Clomburg and Gonzalez 2013); however, 1,3-PD as a by-product hinders 2,3-BD purification in downstream processes because of its similar physicochemical properties with 2,3-BD (Xiu and Zeng 2008; Zhang et al. 2012). Petrov and Petrova (2010) demonstrated 2,3-BD production up to 70 g/L by applying the forced pH fluctuations (ΔpH of 1 every 12 h) with 5 M NaOH; however, 1,3-PD was also produced up to 16.3 g/L. Cho et al. (2015a) reported the highest glycerol-based 2,3-BD titers (115.0 and 131.5 g/L from pure and crude glycerol, respectively) by constructing the double deleted mutant of *K. oxytoca* in which *pduC* (encoding glycerol dehydratase) and *ldhA* (encoding lactate dehydrogenase) were disrupted to block the formation of 1,3-PD and lactic acid, respectively. However, 1,3-PD was still generated when pure glycerol was used (Cho et al. 2015a).

Unlike *Klebsiella* species, *B. amyloliquefaciens*, *Raoultella planticola*, and *Raoultella terreigena* have been reported to produce 2,3-BD from glycerol without 1,3-PD formation (Ripoll et al. 2016; Yang et al. 2013). *B. amyloliquefaciens* revealed 1,3-PD-free 2,3-BD production from crude glycerol at the concentration of 43.1 g/L, whereas supplying beet molasses as a co-substrate was

needed to enhance 2,3-BD production up to 83.3 g/L (Yang et al. 2013). Recently, Ripoll et al. (2016) chose *R. planticola* and *R. terreigena* (previously known as *Klebsiella planticola* and *Klebsiella terreigena*) (Drancourt et al. 2001) as non-pathogenic 2,3-BD producers (belonging to risk group 1) and demonstrated 2,3-BD production at 27.3–33.6 g/L from pure glycerol and crude glycerol in batch fermentation. Use of those 2,3-BD producers lacking the 1,3-PD formation ability can provide advantages in the 2,3-BD purification process; however, 2,3-BD production results by Yang et al. (2013) and Ripoll et al. (2016) are much lower than 2,3-BD titers obtained by *K. pneumoniae* and *K. oxytoca* possessing the 1,3-PD synthesis pathway (Cho et al. 2015a; Petrov and Petrova 2010). In addition, there are limited studies on fermentation conditions favoring 2,3-BD production and on genetically engineered 1,3-PD synthesis-deficient 2,3-BD producers.

Meanwhile, a newly isolated *Raoultella ornithinolytica* B6 was found to have potential as a high 2,3-BD producer from glucose (Kim et al. 2016). According to the completed genome sequence (GenBank accession number CP004142) (Shin et al. 2013), the 1,3-PD synthesis pathway does not exist in *R. ornithinolytica* B6. In this study, based on those previous findings, 1,3-PD-free 2,3-BD production was investigated with *R. ornithinolytica* B6 using pure glycerol and crude glycerol. The effects of the temperature, agitation speed, and pH control strategy on 2,3-BD production were evaluated to find fermentation conditions favoring 2,3-BD production. Notably, controlling the pH with a lower limit of 5.5 was effective to enhance 2,3-BD production. To further increase the 2,3-BD production, the genetic engineering of *R. ornithinolytica* B6 was conducted to increase the carbon flux toward 2,3-BD synthesis by overexpressing the homologous *budABC* genes directly involved in the conversion of pyruvate to 2,3-BD (Kim et al. 2016). To our knowledge, this is the first study on 1,3-PD-free 2,3-BD production from glycerol or crude glycerol with the wild-type *R. ornithinolytica* strain as well as an engineered *Raoultella* strain at high titer, yield, and productivity.

Materials and methods

Microorganism and media

R. ornithinolytica B6 deposited in the Korea Culture Center of Microorganisms (KCCM) as KCCM11176-P was used as the parent strain. The defined medium (Cho et al. 2015a) contained 13.7 g/L K_2HPO_4, 2 g/L KH_2PO_4, 3.3 g/L $(NH_4)_2HPO_4$, 6.6 g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $FeSO_4 \cdot 7H_2O$, 0.001 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L

MnSO$_4$·H$_2$O, 0.01 g/L CaCl$_2$·2H$_2$O, and 50 g/L pure glycerol or crude glycerol as needed. The defined medium supplemented with 5 g/L yeast extract and 10 g/L casamino acid was used as the complex medium.

Pretreatment of crude glycerol

Crude glycerol, denoted as AK, was obtained from Aekyung Ind., Korea, which manufactures biodiesel with an alkali-catalyzed transesterification process. The components of the crude glycerol AK are shown in Table 1. The pretreatment of crude glycerol was conducted according to the method of Moon et al. (2010) with modifications: (1) The glycerol was mixed with distilled water at a ratio of 4:1 (v/v) to reduce the viscosity of the glycerol; (2) the pH was adjusted to pH 1–2 with 5 M HCl; (3) the crude glycerol solution was centrifuged at 6000 rpm for 20 min; (4) the lower phase was obtained, and its pH was adjusted to 7 with 5 M KOH. The composition of the pretreated crude glycerol, denoted as AK-T, is shown in Table 1.

Batch and fed-batch fermentation

For batch cultures in flasks, a single colony from an agar plate was inoculated into the defined medium with 30 g/L glycerol and incubated at 200 rpm in a shaking incubator overnight at 25 or 30 °C. Then, the seed culture was inoculated at 2% (v/v) in a 100-mL Erlenmeyer flask containing 20 mL of defined medium.

All fed-batch fermentations to investigate the effects of the agitation speed, complex nitrogen source, and pH control strategy were performed in a 3-L bioreactor (Fermentec, South Korea) with an initial working volume of 1 L. For seed culture preparation, *R. ornithinolytica* B6 and *budABC* over-expression mutant were inoculated into 100 mL of defined medium in 500-mL flask and cultivated on a rotary shaker at 200 rpm at 25 °C for 12 h. The seed culture (10% v/v) was then inoculated into defined or complex medium as needed. The fed-batch fermentation was carried out at 25 °C, and the initial pH was adjusted 7.0 using 5 M KOH. Air was supplied through sterilized HEPA-VENT (Whatman, Germany) at 1.0 vvm. When the residual glycerol concentration dropped below 20 g/L, 100 mL of a glycerol stock solution (540–600 g/L) was supplied. The influence of the agitation speed was tested at 200, 300, 400, and 500 rpm using the defined medium. After selecting the optimized agitation speed, the effect of pH was evaluated by controlling the pH not to drop below the lower limits (pH 5.5 or 6.0) using 5 M KOH. Finally, the effect of complex nitrogen sources (yeast extract and casamino acid) was tested at the optimum agitation speed and pH control mode.

Overexpression of *budABC*

To improve 2,3-BD production, the plasmid pUC18CM-*budABC* was constructed. The pUC18CM vector harboring chloramphenicol resistance gene was derived from pUC18 (Cho et al. 2013), which contains pMB1 origin and lac promoter. However, there is no *lacI* gene (encoding the *lac* repressor) in pUC18CM; thus, target genes are constitutively expressed without induction. The primers and vectors for overexpression are listed Table 2. The *budABC* genes encoding acetolactate decarboxylase (BudA), acetolactate synthase (BudB), and acetoin reductase (BudC) were amplified from the chromosomal DNA of *R. ornithinolytica* B6 using the primers budA-F and budC-R. Then, the *budABC* fragment was cloned into the *Xba*I and *Hin*dIII restriction sites of pUC18CM. *Escherichia coli* HIT-DH5α (RBC Bioscience Corp., Taiwan) was used to clone these genes. Next, the plasmid pUC18CM-*budABC* was transformed to *R. ornithinolytica* B6 by electroporation. *R. ornithinolytica* B6 harboring the plasmid was cultured with 30 μg/mL of chloramphenicol to maintain the plasmid.

Deletion of the *ldhA* gene

To investigate the influence of lactic acid production on 2,3-BD production, the deletion of the *ldhA* gene (accession number WP_004861243) was conducted with the λ Red recombination method as previously reported by Cho et al. (2015a). The plasmids and primers are listed in Table 2; ldhA-UF and ldhA-UR were used to amplify the upstream regions of *ldhA*. ldhA-DF and ldhA-DR were used to amplify the downstream regions of *ldhA*. Additional PCR using the pTOT-FCF plasmid as a template was performed with the primers ldhA-FU-F and ldhA-FD-R. These PCR products were amplified with the primers ldhA-UP and ldhA-DR using the overlap extension PCR method. The PCR products were transformed into *R. ornithinolytica* B6 harboring pRedET (Gene Bridges, Germany), and chloramphenicol-resistant colonies were selected on LB agar plates containing 30 μg/mL of chloramphenicol at 30 °C. Deletion of the *ldhA* gene from the chromosome was confirmed by the size of the PCR products (1150 bp) obtained using the ldhA-del-F and ldhA-del-R primers. To remove the chloramphenicol-resistant gene from the chromosome, 707-FLPe (Gene Bridges, Germany) was transformed into the cells, and tetracycline-resistant transformants were selected at 30 °C. The 707-FLPe was cured by cultivation at 37 °C. After removal of the chloramphenicol-resistant gene, the resulting mutant was confirmed by PCR (300 bp).

Table 1 Composition of AK and AK-T

Category[a]	Method	Glycerol	
		AK	AK-T
Glycerol, wt%	KSM 1979	68.3	70.23
Water, wt%	ASTM E203	4.4	26.605
Ash, wt%	ASTM ED482	0.7	1.167
MONG[b], wt%	ISO 2464-1973	26.6	2
Methanol[c], mg/kg	GC SGS KR 015 (in-house)	28,000	26,600
Sodium[c], mg/kg	IP 501	10,137.5	9107
Magnesium[c], mg/kg	IP 501	18.8	12
Potassium[c], mg/kg	IP 501	108.8	1410

[a] Analyzed by SGS Testing, South Korea

[b] Matter organic non-glycerol (MONG) [wt%] = 100 − (glycerol [wt%] + water [wt%] + ash [wt%])

[c] Methanol and mineral components were quantified independently with whole crude glycerol for more detail information about crude glycerol composition

Analytical procedures

Cell mass was determined by optical density (OD) at 600 nm using a spectrophotometer (Agilent Cary 60 UV-Vis, USA). Glycerol and lactic acid were analyzed by high-performance liquid chromatography (Agilent HPLC, USA) equipped with an Aminex HPX-87 H column [300 × 7.8 mm] (Bio-Rad, USA). Glycerol (99%, Junsei, Japan) and calcium lactate pentahydrate (Sigma-Aldrich, USA) were used as standard materials for HPLC analysis. The operating conditions of the HPLC were as follows: column temperature, 50 °C; flow rate of mobile phase (5 mM H_2SO_4), 0.6 mL/min; UV detector wavelength, 210 nm.

The concentration of the fermentation products (acetoin, 2,3-BD, ethanol, and acetic acid) was determined with gas chromatography (Shimadzu GC-2010, Japan) equipped with a flame ionization detector and Agilent HP-INNOWAX column [30 m × 0.32 mm, 0.25 μm] (Agilent, USA) under the following conditions: oven temperature, from 50 to 240 °C at a rate of 30 °C/min; injector temperature, 240 °C; detector temperature, 250 °C; carrier gas (N_2) flow rate, 30 mL/min.

Table 2 Plasmids and primers used in this study

Plasmids and primers	Genotype and relevant characteristics	Source of reference
Plasmids		
pUC18CM	Derivative of pUC18, Cm^R	Cho et al. (2013)
pRedET	Derivative of pSC101, Tet^R, temperature-sensitive, carrying lambda red recombinase	GeneBridge
707-FLPe	Derivative of pSC101, Tet^R, temperature-sensitive, containing an FLPe recombinase	GeneBridge
pTOP-FCF	Derivative of pUC, containing an FRT-flanked Cm^R cassette-involved vector	Jung et al. (2013)
Primers[a,b]		
budA-F	5′-TTT<u>TCTAGA</u>ATGACCCATTCTTCTGC-3′ (*Xba*I)	This study
budC-R	5′-TTT<u>AAGCTT</u>TTTAGTTAAAAACCATACCG-3′ (*Hin*dIII)	This study
ldhA-UF	5′-AAGCTTGGGCAGTTAATATCCT-3′	This study
ldhA-UR	5′-*TTCTAGAGAATAGGAACTT*CAAGACTTTTCTCCAG-3′	This study
ldhA-DF	5′-*CTAGAAAGTATAGGAACTTC*TCGTCCTTTCCCTTTTG-3′	This study
ldhA-DR	5′-GTGTCGCCGGGAAT-3′	This study
ldhA-FU-F	5′-AATCACTGGAGAAAAGTCTT*GAAGTTCCTATTCTCTAGAA*-3′	This study
ldhA-FD-R	5′-CAAAAGGGAAAGGACGAG*AAGTTCCTATACTTTCTAG*-3′	This study
ldhA-del-F	5′-TATATACCCGCCAGGCCAAAC-3′	This study
ldhA-del-R	5′-GATAACAGCATTCCTGCCGC-3′	This study

[a] Underlined sequences are restriction enzyme sites

[b] Italic sequences are homologous with the FRT region

About 1.5 mL of culture broth was taken for analysis at each sampling point. Ethanol, acetic acid, 1,3-PD, acetoin, and 2,3-BD were purchased from Sigma-Aldrich (USA) at the highest grade available (>98%).

Results

Selection of *R. ornithinolytica* B6 for 2,3-BD production from glycerol

R. ornithinolytica B6 is able to produce 2,3-BD from not only various sugars (glucose, fructose, galactose, xylose, and sucrose) (Kim et al. 2016) but also from glycerol as the sole carbon source in aerobic conditions (Supplementary Table S1). Interestingly, unlike *Klebsiella* species producing 1,3-PD as a by-product from glycerol under aerobic conditions, *R. ornithinolytica* B6 exhibited 1,3-PD-free 2,3-BD production. The enzymes related to 1,3-PD biosynthesis (glycerol dehydratase and 1,3-PD oxidoreductase) are not identified in the complete genome of *R. ornithinolytica* B6. Therefore, *R. ornithinolytica* B6 was further investigated for 1,3-PD-free 2,3-BD production from glycerol.

Effect of temperature on glycerol fermentation by *R. ornithinolytica* B6

Raoultella species including *R. ornithinolytica* are known to be able to grow well at relatively low temperatures (10–30 °C) (Drancourt et al. 2001). To investigate the effect of temperature on glycerol fermentation, *R. ornithinolytica* B6 was incubated at 25 and 30 °C in flasks. As shown in Supplementary Table S1, 2,3-BD production at 25 °C was 16.86 g/L, whereas only 3.63 g/L of 2,3-BD was produced at 30 °C. The yield of 2,3-BD at 30 °C was much lower than that at 25 °C (0.17 vs. 0.32 g/g) due to the substantial production of ethanol and acetic acid at 30 °C.

Considering the 2,3-BD concentration, productivity, yield, and cell growth, further experiments with *R. ornithinolytica* B6 were performed at 25 °C.

Effect of agitation speed on 2,3-BD production by *R. ornithinolytica* B6

The agitation speed is one of the most important factors influencing 2,3-BD titer, yield, and productivity. In the previous studies on glucose-based 2,3-BD production, optimum agitation speeds were in a range of 200–450 rpm (Cho et al. 2015b; Ji et al. 2009; Park et al. 2013b). However, there was no report on the effect of agitation speed on glycerol-based 2,3-BD production. To investigate the effect of agitation speed on 2,3-BD production from glycerol with *R. ornithinolytica* B6, fed-batch fermentation was tested with four different

agitation speeds: 200, 300, 400, and 500 rpm at 1.0 vvm air flow without pH control. The results are shown in Supplementary Fig. S1. The 2,3-BD concentration and yield at 300 rpm (23.32 g/L, 0.34 g/g) were similar to those of 200 rpm (23.23 g/L, 0.35 g/g); however, the productivity (0.47 g/L/h) was higher at 300 rpm by 67.9% compared with 200 rpm (0.28 g/L/h) (Supplementary Fig. S1a, b). When the fed-batch fermentation was conducted at 400 rpm, the concentration, yield, and productivity of 2,3-BD increased up to 36.40 g/L, 0.39 g/g, and 0.96 g/L/h, respectively (Supplementary Fig. S1c). At 500 rpm, 2,3-BD production was only 7.28 g/L despite high cell growth (Supplementary Fig. S1d). Because 2,3-BD production at 400 rpm showed the highest concentration, yield, and productivity, an agitation speed of 400 rpm was selected for all further studies.

Effect of pH control on 2,3-BD production by *R. ornithinolytica* B6

In addition to the aeration strategy, another important parameter in 2,3-BD production from glycerol is the pH profiles. Petrov and Petrova (2009) demonstrated that pH fluctuations during effective 2,3-BD production from glycerol (with no pH control mode) were caused by alternate acid and 2,3-BD formation as an adaptive response to the pH drop. Petrov and Petrova (2010) further demonstrated that the forced pH fluctuations (ΔpH of 1 every 12 h) were effective to enhance 2,3-BD production up to 70 g/L.

In Fig. 1, when pH was not controlled, the pH fluctuations observed after 20 h of fermentation, but the amplitudes were not significant (ΔpH values less than 0.2). In addition, after 12 h of fermentation, pH rapidly dropped to 5.4 and cell growth was significantly retarded. As the pH further decreased to 4.5 after 38 h of fermentation, 2,3-BD production ceased. To attempt a higher 2,3-BD production by allowing pH fluctuations as well as by preventing acidic conditions, the pH was controlled not to drop below the lower limits which were pH 5.5 or 6.0. In detail, once the pH value decreased to the lower limits (pH 5.5 or 6.0) after 10–12 h of fermentation (Fig. 1a), an alkali solution (5 M KOH) was added to prevent a pH drop below the lower limits. There was no pH control when the pH values were above the lower limits. Notably, the pH fluctuations occurred naturally with much pronounced ΔpH ranges compared to the no pH control mode (Fig. 1a). With the lower limit of pH 5.5, the pH increased up to 6.0–6.3, and then, it dropped to 5.5 again clearly showing several consecutive pH rise and drop profiles with ΔpH values of 0.5–0.8. On the other hand, with the lower limit of pH 6.0, the pH increased up to 6.7–6.9, and it tended to stay above 6.0 with low ΔpH values (0.2–0.3). As expected, cell growth and 2,3-BD production were improved when the pH was controlled above the lower limits (Fig. 1b, c). With the pH control above pH 5.5, 2,3-BD at 60.66 g/L was produced using 154.26 g/L glycerol
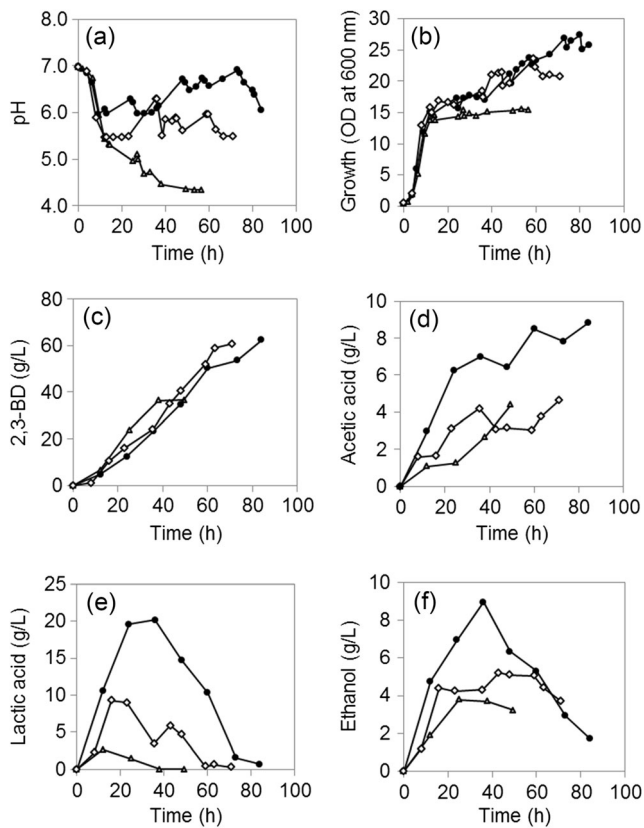
Fig. 1 Time course of **a** pH, **b** cell growth, **c** 2,3-BD, **d** acetic acid, **e** lactic acid, and **f** ethanol under different pH control modes. Fed-batch fermentation was carried with *R. ornithinolytica* B6 at 25 °C and 400 rpm with the defined medium. No pH control (*triangles*), pH control with the lower limit of 5.5 (*diamonds*), pH control with the lower limit of 6.0 (*circles*)
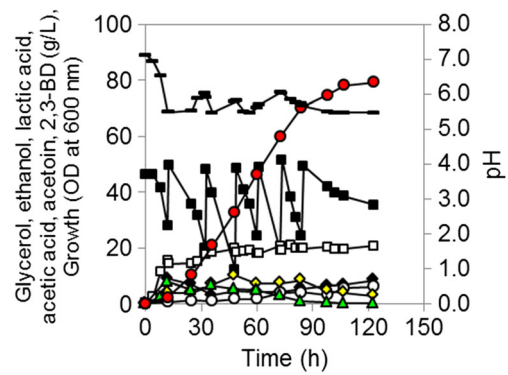

Fig. 2 Fed-batch fermentation of glycerol by *R. ornithinolytica* B6 in the complex medium containing 5 g/L yeast extract and 10 g/L casamino acid at 25 °C and 400 rpm with the lower pH limit of 5.5; glycerol (*black squares*), growth (*white squares*), pH (*line*), 2,3-BD (*red circles*), ethanol (*yellow diamonds*), acetoin (*white circles*), acetic acid (*black diamonds*), lactic acid (*green triangles*)

with 0.39 g/g yield and 0.85 g/L/h productivity. In the case of the pH control above 6.0, the 2,3-BD concentration, yield, and productivity were 62.42 g/L, 0.36 g/g, and 0.74 g/L/h, respectively. The titer of 2,3-BD with the lower pH limit at 6.0 was similar to that at the pH control above 5.5; however, the 2,3-BD yield and productivity at the pH control mode above 6.0 were lower than those at pH 5.5 by 8–13%. This may result from much higher production of by-products, such as acetic acid, lactic acid, and ethanol, in the middle of the fermentation when the pH was controlled at above 6.0 (Fig. 1d–f), which would negatively affect the 2,3-BD productivity and yield. Taking into consideration the 2,3-BD concentration, yield, and productivity as well as the pH fluctuation profiles, all further studies were done at the lower pH limit of 5.5.

Effect of complex nitrogen sources on 2,3-BD production by *R. ornithinolytica* B6

Complex nitrogen sources have been shown to be effective in enhancing cell growth and 2,3-BD production (Cho et al. 2015b; Jung et al. 2012). To investigate the effect of complex nitrogen sources on 2,3-BD production, yeast extract (5 g/L)

and casamino acid (10 g/L) were added to the medium. Fed-batch fermentation was conducted under the following selected conditions: an agitation speed of 400 rpm and a lower pH limit of 5.5. As shown in Fig. 2 and Supplementary Table S2, 2,3-BD production was much enhanced up to 79.25 g/L compared to 2,3-BD titer obtained with the defined medium (60.66 g/L). The addition of complex nitrogen sources appeared to be effective in prolonging the fermentation time up to 123 h. Although the overall productivity with the complex medium (0.64 g/L/h) was lower than that with the defined medium (0.85 g/L/h) likely due to the lag phase and prolonged fermentation time, the maximum productivities in the middle of fermentation were similar with the defined medium and the complex medium (1.01–1.03 g/L/h).

Enhancement of 2,3-BD production by overexpression of the *budABC* genes

Even though the 2,3-BD titer was increased by adding complex nitrogen sources, the production of lactic acid was up to 7.79 g/L after 12 h, and acetic acid production was increased to 8.38 g/L at the end of fermentation (Fig. 2). The decrease of lactic acid production by deleting the lactate dehydrogenase gene (*ldhA*) has been shown to positively affect 2,3-BD production (Jung et al. 2012). Therefore, a *ldhA* deletion mutant was constructed with the expectation that the carbon and NADH flux to 2,3-BD synthesis would be increased by blocking the lactic acid production pathway. However, 2,3-BD titer (41.88 g/L), yield (0.27 g/g), and productivity (0.51 g/L/h) were much lower than those of the wild type (Supplementary Fig. S2). Moreover, ethanol production was significantly increased up to 22.29 g/L at 40 h and then decreased to 15.17 g/L after 82 h of fermentation. This result implies that the increased carbon and redox power in the *ldhA* deletion mutant was likely utilized in ethanol synthesis. Interestingly, the pH fluctuation was marginal compared to

the wild type, indicating that the pH fluctuation during the fermentation of *R. ornithinolytica* B6 was likely related to lactic acid profiles. This insignificant pH fluctuation might also negatively influence the 2,3-BD production.

Alternatively, a *budABC* overexpression mutant was constructed to increase the carbon flux toward the 2,3-BD synthesis and consequently to decrease the carbon flux to acetyl-CoA, an intermediate of both acetic acid and ethanol. The BudABC enzymes encoded by *budABC* are directly involved in the conversion of pyruvate to 2,3-BD: α-Acetolactate synthase (BudB) (accession number AGJ85610) catalyzes the synthesis of α-acetolactate from 2 mol of pyruvate; α-acetolactate decarboxylase (BudA) (accession number AGJ85611) converts α-acetolactate to acetoin, and acetoin is converted to 2,3-BD by acetoin reductase (BudC) (accession number AGJ85609). The overexpression of these genes (*budABC*) was confirmed by SDS-PAGE (Supplementary Fig. S3). There was no difference in growth and product profiles between the parent strain and *R. ornithinolytica* B6 harboring the empty vector (pUC18CM) (data not shown).

When the fed-batch fermentation of *R. ornithinolytica* B6 (pUC18CM-*budABC*) was conducted, the pH fluctuation was clearly observed (Fig. 3), and the 2,3-BD production was enhanced up to 89.45 g/L with the yield of 0.41 g/g and the productivity of 0.75 g/L/h. The 2,3-BD production performance was higher than the results obtained with the wild-type strain (Supplementary Table S2). As expected, the production of acetic acid and ethanol was decreased.

Crude glycerol utilization for 2,3-BD production with the *budABC* overexpression mutant

Crude glycerol, a by-product from biodiesel processes, contains glycerol, water, and impurities (methanol, matter organic

Fig. 3 Fed-batch fermentation of glycerol by *R. ornithinolytica* B6 (pUC18CM-*budABC*) in the complex medium at 25 °C and 400 rpm with the lower pH limit of 5.5; glycerol (*black squares*), growth (*white squares*), pH (*line*), 2,3-BD (*red circles*), ethanol (*yellow diamonds*), acetoin (*white circles*), acetic acid (*black diamonds*), lactic acid (*green triangles*)

non-glycerol (MONG), inorganic salts, and ash) (Luo et al. 2016). To investigate the feasibility of 2,3-BD production from crude glycerol, *R. ornithinolytica* B6 was cultivated in flasks using crude glycerol (AK) and the corresponding pretreated crude glycerol (AK-T). The compositions of the AK and AK-T are shown in Table 1. Pretreatment was done according to the method of Moon et al. (2010) by which soap and free fatty acids are known to be removed.

When AK was supplied as the sole carbon source for *R. ornithinolytica* B6 (pUC18CM-*budABC*) under the selected fermentation conditions (25 °C, 400 rpm, complex medium, and a lower pH limit of 5.5) (Fig. 4a), 65.41 g/L of 2,3-BD was produced after 168.52 g/L of glycerol consumption during 86.5 h at the yield of 0.39 g/g and the productivity of 0.76 g/L/h (Supplementary Table S2). The produced 2,3-BD titer was approximately 73.0% of that obtained with pure glycerol using *R. ornithinolytica* B6 (pUC18CM-*budABC*). This low 2,3-BD production with crude glycerol could be the result of the accumulation of inhibitory impurities (existing in the crude glycerol) due to the repeated feeding of crude glycerol. Another possible reason could be the marginal pH fluctuations shown in Supplementary Fig. S4a. The pH of the crude glycerol AK generated by an alkali-catalyzed biodiesel process was in the range of pH 10–11. Therefore, AK appeared to act like an alkali solution, and the pH did not rapidly decrease during the fermentation. Lactic acid production was also low (less than 1 g/L) in agreement with the low pH drop profiles.

In the case of adding AK-T (the pretreated AK at pH 7), the 2,3-BD concentration and yield were enhanced up to 78.10 g/L and 0.42 g/g, respectively, compared to the case of using crude glycerol AK (Fig. 4b and Supplementary Table S2). The production of 2,3-BD was prolonged up to 126 h probably due to the removal of impurities through the pretreatment. Furthermore, pH fluctuation and lactic acid formation/consumption were clearly observed (Supplementary Fig. S4).
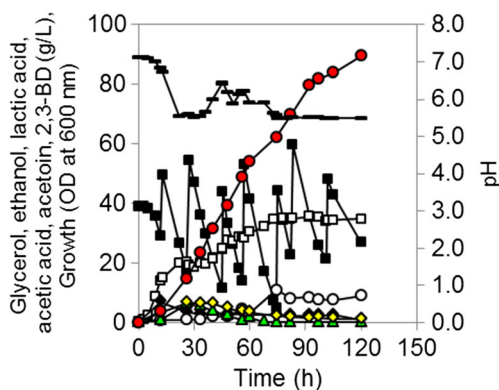
Discussion

Raoultella species are generally found in aquatic environmental samples, insects, and fishes. Although *R. ornithinolytica* strains have been isolated from clinical samples, human infections by *R. ornithinolytica* are known to be extremely rare (Sun et al. 2015). Given the facts that *R. ornithinolytica* B6 grows very well at low temperature (25 °C) and *R. ornithinolytica* B6 did not grow at human normal body temperature (37 °C) as shown in the previous our report (Kim et al. 2016), the possibility of human infection by *R. ornithinolytica* B6 is expected to be extremely low.

R. ornithinolytica B6 has some characters distinguished from other 2,3-BD producers. First, there is no glycerol dehydratase in the genome of *R. ornithinolytica* B6. This
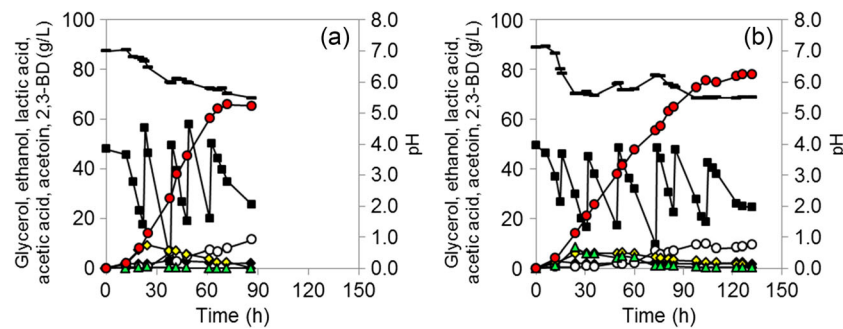
Fig. 4 Fed-batch fermentation using **a** crude glycerol, AK and **b** pretreated crude glycerol AK-T by *R. ornithinolytica* B6 (pUC18CM-*budABC*) in complex medium at 25 °C and 400 rpm with the lower pH limit of 5.5; glycerol (*black squares*), pH (*line*), 2,3-BD (*red circles*), ethanol (*yellow diamonds*), acetoin (*white circles*), acetic acid (*black diamonds*), lactic acid (*green triangles*)

might support the lack of 1,3-PD formation from glycerol. Because 1,3-PD-free 2,3-BD production from glycerol provides a significant advantage in purification processes of 2,3-BD, *R. ornithinolytica* B6 was selected for 2,3-BD production from glycerol. Second, the optimum temperature (25 °C) for 2,3-BD production is relatively low compared to the previous studies on 2,3-BD production from glycerol: *K. pneumoniae* (Petrov and Petrova 2010) and *B. amyloliquefaciens* (Yang et al. 2013) were cultivated at 37 °C; *K. oxytoca* was incubated at 30 °C (Cho et al. 2015a), and *R. terrigena* and *R. planticola* showed higher 2,3-BD yield and productivity at 30 °C than 26 °C (Ripoll et al. 2016).

After selecting the optimum temperature through flask cultivation test, other important operation variables such as

aeration condition and pH control mode were investigated in fed-batch fermentation for improving 2,3-BD production of *R. ornithinolytica* B6. Titer and productivity of 2,3-BD are generally increased with agitation speed, but too much oxygen supply tends to stimulate cell mass formation rather than 2,3-BD production (Cho et al. 2015b; Kim et al. 2016). To find the optimum agitation speed for glycerol-based 2,3-BD production by *R. ornithinolytica* B6, fed-batch fermentation was tested under different agitation speeds (200, 300, 400, and 500 rpm) and 400 rpm was selected as the optimum agitation speed. Regarding pH control, because the 2,3-BD production was ended after 38 h of fermentation under no pH control mode probably due to a significant pH drop (Fig. 1c), pH control was necessary to prevent acidic conditions. Three different pH control strategies were investigated: (i) no pH

Table 3 Comparison of 2,3-BD production from glycerol between the representative previous reports and this study

Strain	Carbon source	Culture mode	2,3-BD			Reference
			Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	
Klebsiella species[a]						
K. pneumoniae G31	Pure glycerol	Fed-batch	49.2	0.36	0.18	Petrov and Petrova (2009)
K. pneumoniae G31	Pure glycerol	Fed-batch	70.0	0.39	0.47	Petrov and Petrova (2010)
K. oxytoca M3[b]	Pure glycerol	Fed-batch	115.0	0.39	1.01	Cho et al. (2015a)
	Crude glycerol	Fed-batch	131.5	0.44	0.84	Cho et al. (2015a)
Non-*Klebsiella* species[c]						
B. amyloliquefaciens	Crude glycerol	Fed-batch	43.1	0.38	0.45	Yang et al. (2013)
R. planticola	Pure glycerol	Batch	30.5	0.38	0.30	Ripoll et al. (2016)
	Crude glycerol	Batch	27.5	0.31	0.23	Ripoll et al. (2016)
R. terrigena	Pure glycerol	Batch	27.3	0.30	0.38	Ripoll et al. (2016)
	Crude glycerol	Batch	33.6	0.38	0.35	Ripoll et al. (2016)
R. ornithinolytica B6	Pure glycerol	Fed-batch	79.25	0.40	0.64	This study
R. ornithinolytica B6 (pUC18CM-*budABC*)	Pure glycerol	Fed-batch	89.45	0.41	0.75	This study
	Crude glycerol	Fed-batch	65.41	0.39	0.76	This study
	Pretreated Crude glycerol	Fed-batch	78.10	0.42	0.59	This study

[a] Possessing 1,3-PD synthesis pathway

[b] Deficient in 1,3-PD synthesis ability

[c] *pduC* and *ldhA*-deleted mutant of *K. oxytoca* M1

control, (ii) pH control with the lower limit of 5.5, and (iii) pH control with the lower limit of 6.0. The reason that pH was not strictly controlled at a certain fixed level was to permit pH fluctuations which are known to positively affect 2,3-BD production. Petrov and Petrova (2010) developed the method of "forced pH fluctuations" to stimulate glycerol consumption and consequently 2,3-BD production. The pH was intentionally fluctuated by consecutively adding 5 M NaOH to raise pH with ΔpH of 1 every 12 h. As a result, the final 2,3-BD concentration during 150 h of fermentation was 70.0 g/L, which was 1.33-fold higher than that of no pH control case (52.5 g/L). In this study, instead of making intentional pH fluctuations, the lower limit of pH was set to investigate pH profiles and 2,3-BD production. Interestingly, without intentional forced pH fluctuations, the pronounced natural pH fluctuations were established when the lower limit of the pH was set at 5.5 or 6.0. This pH control strategy was effective in increasing 2,3-BD concentration by 1.67-fold (with the pH control above 5.5) and 1.71-fold (with the pH control above 6.0) compared to the no pH control mode. Moreover, we found that the pH fluctuations were related to the production of lactic acid through *ldh* deletion experiment (Supplementary Fig. S2). The pH fluctuations during 2,3-BD production were likely caused by alternate lactic acid and 2,3-BD formation as an adaptive response to the pH drop. Similar pH profiles have been observed with butanol-producing clostridia fermentation (e.g., pH drop due to acid production and then pH rise by acid reassimilation to butanol) (Youn et al. 2016). Park et al. (2013a) also reported that, through analysis of the metabolic flux distribution in *K. oxytoca*, the lactic acid flux at pH 5.5 was lower than that at pH 7, while the flux toward 2,3-BD synthesis at pH 5.5 was higher than that at pH 7.0. Therefore, consecutive pH drop and rise observed in this study appears to be associated to the metabolic flux control mechanism in *R. ornithinolytica* B6: When pH dropped to 5.5 (at the lower limit of pH 5.5), the metabolic flux toward 2,3-BD formation might be increased and consequently pH rise occurred.

Table 3 shows the 2,3-BD production using glycerol (pure or crude) as the sole carbon source from previous reports and this study. Petrov and Petrova (2010) and Cho et al. (2015a) accomplished high production of 2,3-BD from glycerol using *K. pneumoniae* and *K. oxytoca*, respectively; however, those *Klebsiella* species possess the 1,3-PD synthesis pathway and tend to produce 1,3-PD as a by-product. Yang et al. (2013) showed 2,3-BD production from glycerol using *B. amyloliquefaciens* in which 1,3-PD synthesis was not detected; yet, the 2,3-BD titer was much lower than the results of Petrov and Petrova (2010) and Cho et al. (2015a). Recently, Ripoll et al. (2016) reported *R. terrigena* and *R. planticola* as 2,3-BD producers from glycerol and accessed the optimum temperature, initial glycerol concentrations in batch fermentation, and yeast extract concentrations; however, the 2,3-BD production was relatively low (27.3–33.6 g/L), and further

investigation such as pH control strategies and mutant construction was not reported. In this study, a high production of 2,3-BD without 1,3-PD formation was achieved by (i) utilizing *R. ornithinolytica* B6 lacking the 1,3-PD synthesis pathway, (ii) generating pronounced natural pH fluctuations simply by controlling the lower pH limit at 5.5, and (iii) constructing the *budABC* gene overexpressing mutant. The 2,3-BD titer, yield, and productivity values obtained in this study are the highest values among the 1,3-PD biosynthesis-free 2,3-BD producers (Table 3). Consequently, 2,3-BD production from glycerol by *R. ornithinolytica* B6 is promising with the following advantages: no possibility of 1,3-PD production and a simple pH control strategy compared to *Klebsiella* species previously known as the best 2,3-BD producers.

In conclusion, *R. ornithinolytica* B6 was shown as a promising 2,3-BD producer using glycerol as a carbon source. *R. ornithinolytica* B6 has no 1,3-PD synthesis pathway; therefore, the downstream cost for separation/purification of 2,3-BD in industrial processes can be reduced. Enhancement of 2,3-BD production was achieved by optimizing the fermentation conditions (temperature, agitation speeds, and pH control) and metabolically engineered *R. ornithinolytica* B6. Subsequently, *R. ornithinolytica* B6 (pUC18CM-*budABC*) effectively produced 2,3-BD using pure glycerol and crude glycerol up to 89.45 and 78.10 g/L, respectively, providing a feasible 1,3-PD-free 2,3-BD production process.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

Ethical approval   This article does not contain any studies with human participants or animals performed by any of the authors.

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