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Metabolic engineering of *Raoultella ornithinolytica* B6 for enhanced production of 2,3-butanediol

2,3-Butanediol 생산을 위한 *Raoultella ornithinolytica* B6의 대사공학적 연구

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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Advisor: Professor Jin-Ho Seo

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Abstract

Metabolic engineering of *Raoultella ornithinolytica* B6 for enhanced production of 2,3-butanediol

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2,3-Butanediol (2,3-BD) is a platform chemical with wide ranges of industrial applications. Numerous studies have been reported with regard to metabolic engineering of microorganisms able to produce 2,3-BD and optimization of fermentation processes in an effort to improve 2,3-BD production. To overcome limitation in industrial applications caused by the pathogenicity of previously reported 2,3-BD producers such as *Klebsiella pneumoniae* and *K. oxytoca*, a non-pathogenic 2,3-BD producing bacterium, *Raoultella ornithinolytica* B6 was isolated from a soil sample of Baegun Mountain in Korea. The B6 strain produces 2,3-BD as a main product by using glucose, galactose, fructose, xylose, sucrose and glycerol as carbon sources.

Notable physiological characteristics of *R. ornithinolytica* B6 were observed. Cell growth and 2,3-BD production were higher at 25 ^{o}C than those at 30 ^{o}C. The B6 strain has no gene encoding glycerol dehydratase, which converts glycerol to 3-hydroxypropionaldehyde, an intermediate of 1,3-propanediol (1,3-PD)

biosynthetic pathway, hence the B6 strain cannot produce 1,3-PD, which may cause difficulty in 2,3-BD purification processes.

2,3-BD production by *R. ornithinolytica* B6 was improved by metabolic engineering and optimizing fermentation conditions including pH and aeration. First, production of 2,3-BD from glucose was conducted. For optimizing fermentation conditions, pH was controlled by the two-stages; initial pH was set at 7.0 and then the acidity was maintained at pH 5.5 after naturally decreasing to pH 5.5. As a result, 2,3-BD production was increased by a 1.5-fold compared with no pH-control. Optimum agitation speed for 2,3-BD production was also investigated for 300, 400 and 500 rpm. The highest concentration of 2,3-BD (68.3 g/L) was obtained at 400 rpm. Further improvement of 2,3-BD production in titer (112.2 g/L) and productivity (1.35 g/L/h) was achieved by overexpressing the homologous *budABC* genes directly involved in the conversion of pyruvate to 2,3-BD. Second, fermentation conditions for using glycerol as a carbon source were optimized for efficient 2,3-BD production. By evaluating the effects of agitation speed, and pH control strategy, optimum fermentation conditions for 2,3-BD production were found to be 400 rpm, and pH control with lower limit of 5.5. Notably, significant pH fluctuations which positively affected 2,3-BD production were generated by simple control of the lower pH limit at 5.5. In a fed-batch fermentation under those conditions, *R. ornithinolytica* B6 produced 79.3 g/L 2,3-BD, and a further enhancement of 2,3-BD production (89.5 g/L) was achieved by overexpressing the *budABC* genes. When pretreated crude glycerol was used as a sole carbon source, the engineered *R. ornithinolytica* B6 produced 78.1 g/L 2,3-BD with 0.42 g/g of yield and 0.62 g/L/h of productivity. The 2,3-BD titer, yield and productivity were the highest values among 1,3-PD synthesis-deficient 2,3-BD producers.

This study demonstrates *R. ornithinolytica* B6 as a promising 2,3-BD producer, which can produce 2,3-BD at high concentrations from glucose and glycerol.

Keywords : 2,3-butanediol, *Raoultella ornithinolytica*, fed-batch fermentation, metabolic engineering, glycerol

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Chapter 1

Literature review

1.1. 2,3-Butanediol

2,3-Butanediol (2,3-BD) is called 2,3-butylene glycol and 1,3-dimethylene glycol, and composed of four carbons and two hydroxyl groups with 90.1 g/mol of molecular weight. 2,3-BD exists in three stereoisomers (dextro-, levo-, and meso- forms) (Zeng & Sabra, 2011) and their boiling point range is 177 - 182 °C (Voloch et al., 1985).

2,3-BD is a typical platform chemical and has wide industrial applications; manufacture of printing inks, softening, perfumes (Leja et al., 2006), moisturizer (Simion et al., 2005) and plant growth hormone (Ryu et al., 2003). Levo-2,3-BD is used as an antifreeze due to its low freezing point, - 60°C. 2,3-BD can be converted to methyl ethyl ketone (MEK), a liquid fuel additive and organic solvent for resins and also to butadiene, a monomer for polyesters and polyurethanes by dehydration. The dehydrogenation of 2,3-BD generates acetoin and diacetyl, which are used as food flavors. The esters of butanediol are effective plasticizer for thermoplastic polymers. (Leja et al., 2006; Voloch et al., 1985; Zeng & Sabra, 2011).

2,3-BD can be chemically synthesized based on petroleum. Because of unstable petroleum supply and increasing environmental concerns, biological production of 2,3-BD from renewable resources is an alternative to conventional chemical processes. 2,3-BD can be produced by fermentation of hexoses, pentoses, disaccharides (Zeng & Sabra, 2011), and glycerol by microorganisms, thus cellulosic biomass is also available as feedstock for 2,3-BD production. Then, 2,3-BD is recovered by steam stripping, pervaporation, liquid-liquid extraction, and solvent extraction (Xiu & Zeng, 2008).

1.2. Biological production of 2,3-butanediol

Many bacteria have been reported to produce 2,3-butanediol (2,3-BD) at high concentrations (Table 1) including *Bacillus amyloliquefaciens* (Yang et al., 2012b), *Serratia marcescens* (Zhang et al., 2010b), *Enterobacter aerugenes* (Jung et al., 2012), *Klebsiella pneumoniae* (Ma et al., 2009; Tsvetanova et al., 2014), and *K. oxytoca* (Cho et al., 2015b; Ji et al., 2009; Kim et al., 2013a). Among those 2,3-BD producing bacteria, *K. oxytoca*, *K. pneumoniae* and *E. aerogenes* are considered as promising 2,3-BD producers. Those bacteria produce 2,3-BD through mixed acid fermentation. The pathways from glucose to 2,3-BD and by-products are shown in Figure 1.

Because the 2,3-BD synthesis consumes NADH, blocking of the competitive by-products pathways (e.g., lactic acid and ethanol) for NADH and carbon flux were generally conducted to increase 2,3-BD production. The *ldhA* (encoding lactate dehydrogenase) deleted mutant of *E. aerogenes* produced 118.1 g/L 2,3-BD (Jung et al., 2012). Guo et al. (2014) reported that the deletion of lactate dehydrogenase and aldehyde dehydrogenase for disrupting lactic acid production and ethanol production in *K. pneumoniae* dramatically improved 2,3-BD production up to 116 g/L. Meanwhile, when glycerol is used as a sole carbon source for 2,3-BD production, *Klebsiella* species tend to produce 1,3-propanediol (1,3-PD), which is also a NADH-consuming by-product. Consequently, Cho et al. (2015a) constructed the double deletion mutant of *K. oxytoca* in which glycerol dehydratase and lactate dehydrogenase were inactivated, a key enzymes for the pathway of 1,3-PD and lactic acid, respectively. As a result, 131.5 g/L of 2,3-BD was produced from crude glycerol.

Another metabolic engineering strategy is overexpression of *budB*, *budA*, and *budC* encoding acetolactate synthase, acetolactate decarboxylase, and acetoin reductase/butanediol dehydrogenase (AR/BDH), respectively. Kim et al. (2012) reported high 2,3-BD production (101.5 g/L) with all of the genes involved in 2,3-BD production (*budABC*) overexpressed *K. pneumoniae*. Using *K. oxytoca*, 142.5 g/L of 2,3-BD was achieved by overexpression of *budC* only (Cho et al., 2015b). However, because most 2,3-BD producing organisms belong to the biosafety level 2, the application of a GRAS (generally regarded as safe), *Saccharomyces cerevisiae* has been attempted (Table 2).

Table 1. Microbial 2,3-BD production with high concentration using variable carbon sources

Strain	Substrate	Description	2,3-BD			Reference
			Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	
Klebsiella pneumoniae	Glucose	Wild type	150.0	0.43	4.21	Ma et al. (2009)
	Glucose	*budABC* overexpression	101.5	0.34	2.54	Kim et al. (2012)
	Glycerol	Wild type	70.0	0.39	0.47	Petrov and Petrova (2010)
Klebsiella oxytoca	Glucose	*budC* overexpression	142.5	0.42	1.47	Cho et al. (2015b)
	Crude glycerol	*ΔldhA ΔpduC*	131.5	0.44	0.84	Cho et al. (2015a)
Enterobacter aerogenes	Glucose	Wild type	110.0	0.49	5.4	Zeng et al. (1991)
	Glucose	*ΔldhA*	118.1	0.42	3.2	Jung et al. (2012)
Serratia marcescens	Sucrose	*ΔswrW*	152.0	0.41	2.67	Zhang et al. (2010a)
Penibacillus polymyxa	Sucrose	Wild type	111.0		2.05	Hassler et al. (2012)
Bacillus amyloliquefaciens	Glucose	Wild type	61.4	0.38	1.71	Yang et al. (2012b)
	Waste glycerol	pMA5-*acr*-HapII-*dhaD*-P_{bdhA}-*alsR*	102.3	0.44	1.16	Yang et al. (2015)

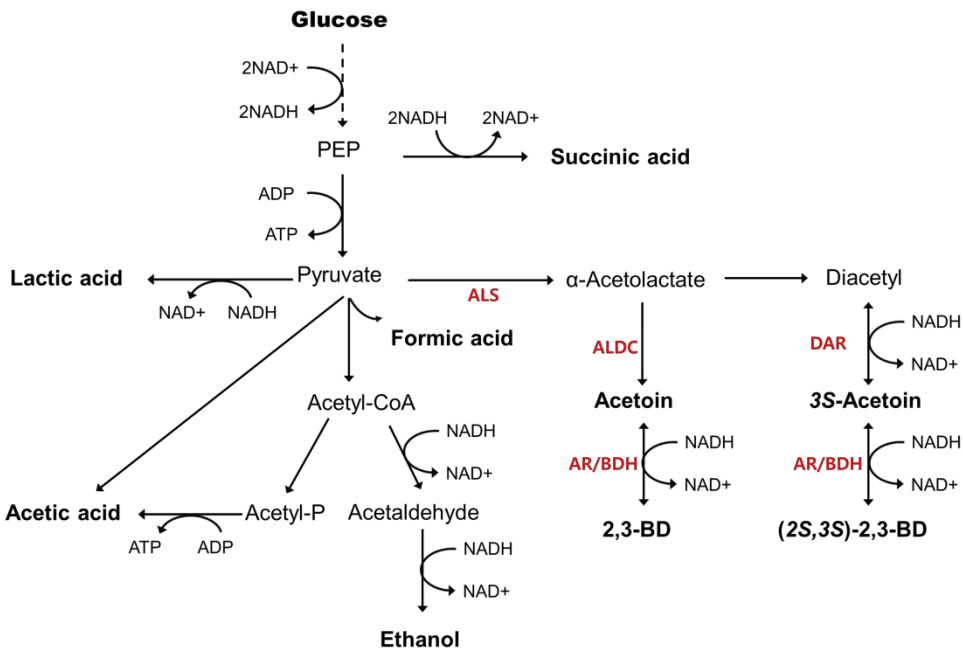

Figure 1. 2,3-BD synthesis pathway in bacteria; ALS, α-acetolactate synthase; ALDC, α-acetolactate decarboxylase; BDH, butanediol dehydrogenase; DAR, diacetyl reductase.

Wild type *S.cerevisiae* produces 2,3-BD at low concentration, yield and productivity compared with 2,3-BD producing bacteria, thus *S. cerevisiae* was not considered as a 2,3-BD producer. However, because its genetic information is fully understood and the tools for genetic engineering are well developed, 2,3-BD production of *S. cerevisiae* was dramatically improved by metabolic engineering.

Because ethanol was produced dominantly in fermentation of *S. cerevisiae*, blocking of the ethanol pathway is essential for efficient 2,3-BD production of *S. cerevisiae*. Kim et al. (2013b) constructed the Pdc-deficient *S. cerevisiae* by disruption of *PDC1* and *PDC5* and hence improved glucose consumption by a point mutation on *MTH1* (encoding transcriptional regulator involved in glucose sensing) caused by evolutionary engineering. Finally, 96.2 g/L 2,3-BD was achieved by introduction of the *alsS* (encoding acetolactate synthase) and *alsD* (encoding acetolactate decarboxylase) genes of *B. subtilis*, and overexpression of the endogenous *BDH1* gene (encoding butanediol dehydrogenase). The mutant was named as *S. cerevisiae* BD4. Moreover, through introducing the *XYL1*, *XYL2*, and *XYL3* genes of *Scheffersomyces stipitis* to *S. cerevisiae* BD4, 43.6 g/L of 2,3-BD was produced from xylose under a fed-batch fermentation (Kim et al., 2014b).

However, the metabolic shift of glucose to 2,3-BD instead of ethanol in *S. cerevisiae* causes excess of intracellular NADH. As a result, glycerol accumulated. To solve this problem, Kim et al. (2015b) introduced the *Lactococcus lactis* water-forming NADH oxidase gene (*noxE*) to Pdc-deficient *S. cerevisiae*. Consequently, the 2,3-BD yield was increased and glycerol yield was successfully decreased.

Table 2. 2,3-BD production of metabolically engineered *S. cerevisiae*

Substrate	Description	2,3-BD			Reference
		Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	
Glucose	*Δpdc1Δpdc5*, expression of *alsSD* (*B. subtilis*), overexpression of endogenous *BDH1*	96.2	0.28	0.39	Kim et al. (2013b)
Xylose	*Δpdc1Δpdc5*, expression of *alsSD* (*B. subtilis*) *and XYL123* (*S. stipitis*) overexpression of endogenous *BDH1*	43.6		0.20	Kim et al. (2014b)
Glucose	*Δpdc1Δpdc5 Δpdc6*, expression of *alsSD* (*B. subtilis*) *and noxE* (*S. stipitis*), overexpression of endogenous *BDH1*	32.4	0.36		Kim et al. (2015b)
Glucose, Galactose	*Δpdc1Δpdc5 Δpdc6*, *MTH1* introduction, expression of *alsSD* (*B. subtilis*), overexpression of endogenous *BDH1*	100	0.35	0.31	Lian et al. (2014)
Glucose	*Δadh1-5 Δgpd1 Δgpd2*, expression of *alsSD* (*B. subtilis*), and *noxE* (*L. lactis*)	72.9	0.41	1.43	Kim and Hahn (2015)

In microbial fermentations, temperature, pH, and aeration are considered as important factors. These factors strongly depend on microorganisms and substrates used. For high production of 2,3-BD, the optimization of fermentation condition is inevitable.

An optimum temperature for 2,3-BD production varies, depending on microorganisms; 37 °C for *K. pneumoniae* (Petrov & Petrova, 2010) and *B. amyloliquefaciens* (Yang et al., 2013); 30 °C for *K. oxytoca* (Cho et al., 2015a), *R. terrigena,* and *R. planticola* (Ripoll et al., 2016).

The second factor affecting 2,3-BD production is pH. Ma et al. (2009) achieved the highest 2,3-BD concentration (150 g/L) from glycerol using *K. pneumoniae* through optimizing a feeding strategy with pH control. They applied the two-stage pH control. The culture was started at pH 7.0 then maintained at 6.0 after pH decreased. Yang et al. (2015) also employed a two-stage pH control strategy for *B. amyloliquefaciens*. The most interesting pH control strategy is "forced pH fluctuation". Petrov and Petrova (2009) noticed that 2,3-BD production depends on the pH fluctuations. Consequently, they designed the "forced pH fluctuation method" and optimized the ΔpH and time interval; pH was intentionally fluctuated by consecutively adding 5N NaOH to raise pH with ΔpH of 1 every 12 hours (Petrov & Petrova, 2010).

The most important factor in 2,3-BD fermentation is aeration. According to Jansen et al. (1984) and Sablayrolles and Goma (1984), high oxygen supply improved both cell growth and 2,3-BD yield. Because the control of agitation speed is effective and simple for control of dissolved oxygen concentration (Ji et al., 2009), finding an optimum agitation and switching agitation speed has

been investigated. The improvement of 2,3-BD production using the switching agitation speed was reported to be an effective method for enhancing 2,3-BD production by *Klebsiella* (Ji et al., 2009; Park et al., 2013b) and *Bacillus* (Yang et al., 2015) species.

1.3. Glycerol as a carbon source for microorganisms

Crude glycerol generated during biodiesel production is a promising renewable resource (Clomburg & Gonzalez, 2013). In biodiesel production, crude glycerol is generated as a byproduct about 10% (w/w) of biodiesel produced. Because of the depletion of fossil sources and the increase in environmental concerns, the production of biodiesel as an alternative energy source has increased. Consequently, the crude glycerol production also increased and its price dramatically decreased (Yang et al., 2012a).

Crude glycerol is composed of water, glycerol, matter organic non-glycerol (MONG), methanol, salts, and ash (Luo et al., 2016; Moon et al., 2010). Although the composition varies with biodiesel manufacturers and resources for biodiesel production, glycerol is a main component. The effort for biological conversion of this low value residue into value-added products is increasing (Chatzifragkou et al., 2010; Posada et al., 2012). Engineered *Escherichia coli* could produce 3-hydroxypropionic acid (Jung et al., 2014), 1,2-propanediol (Clomburg & Gonzalez, 2011), ethanol (Shams Yazdani & Gonzalez, 2008), and lactic acid (Mazumdar et al., 2010) from glycerol with appropriate genetic modifications. *Propionibacterium acidipropionici* can produce propionic acid (Barbirato et al., 1997) and *Clostridium pasteurianum* produce n-butanol from glycerol (Ahn et al., 2011; Lin et al., 2015; Lipovsky et al., 2016). Succinic acid is produced from glycerol by engineered *Yarrowia lipolytica* (Gao et al., 2016) and *Actinobacillus succinogenes* (Roca et al., 2010). The bioconversion of glycerol to 1,3-propanediol (1,3-PD) is conducted by *K. pneumoniae* (Aquino de Souza et al., 2014; Chen et al., 2016) and *K. oxytoca* (Yang et al., 2007).

Because *K. pneumoniae* and *K. oxytoca* have pathways for both 2,3-BD and 1,3-PD, there are studies about co-production of 2,3-BD and 1,3-PD and the main product depends on fermentation conditions such as pH and aeration (Biebl et al., 1998; Metsoviti et al., 2012; Petrov & Petrova, 2010; Sattayasamitsathit et al., 2011; Yen et al., 2014). 1,3-PD can be produced from glycerol by only two enzymes, glycerol dehydratase and 1,3-PD dehydrogenase using NADH as a cofactor. It means 1,3-PD production is an effective pathway for redox balance in comparison to other products during glycerol fermentation of bacteria harboring the 1,3-PD pathway. Therefore, 1,3-PD is a competitive metabolite for NADH and carbon flux in 2,3-BD production for glycerol fermentation. Because 1,3-PD has similar physicochemical properties to 2,3-BD, 1,3-PD is impediment in 2,3-BD production downstream process (Xiu & Zeng, 2008; Zhang et al., 2012). Therefore, blocking of 2,3-BD synthesis (Zhang et al., 2012) or 1,3-PD synthesis (Cho et al., 2015a; Kumar et al., 2016) is conducted on purpose.

1.4. The *Raoultella* genus

Drancourt et al. (2001) suggested reclassification of several *Klebsiella* species including *K. terrigena, K. planticola, K. trevisanii and K. ornithinolytica* as *Raoutella* genus based on carbon utilization, comparison of housekeeping gene sequences (*rpoB* and 16S rDNA). These strains are found in environmental samples. *Raoultella* species are Gram-negative, non-motile and rod and can grow at relatively low temperatures, 10°C. *R. ornithinolytica, R. planticola, R. terrigena* can grow even at 5 °C (Kimura et al., 2014). They can produce organic acids and 2,3-BD from glucose.

There are several studies about the applicability of *Raoultella* species. *R. planticola* has been investigated for the production of pullulanase, a debranching enzyme hydrolyzing pullulan and branched polysaccharides (Hii et al., 2012) and for degradation of lipids in contaminated wastewater. (Sugimori et al., 2013). *R. terrigena* was used for studies on the 2,3-BD producing related genes, *budABC* (Blomqvist et al., 1993) and regulatory gene *budR* (Mayer et al., 1995). Recently, Ripoll et al. (2016) reported 2,3-BD production by *R. terregina* and *R. planticola* from glycerol and crude glycerol. They investigated the effects of temperature and medium composition on 2,3-BD yield and productivity. The polysaccharide-protein complex and Tris-peptide complex from *R. ornithinolytica* have been shown to have antifungal-anticancer effects (Fiolka et al., 2013) and antitumour-apoptotic effects (Fiolka et al., 2015), respectively. Kimura et al. (2014) reported a new speices, *R. electrica*, isolated from activated sludge. However, the use of *Raoultella* species for biological production of chemicals through fed-batch fermentation as well as metabolic engineering has not been reported to date.

1.5. Objectives of the dissertation

This study aims to expand the spectrum of 2,3-butanediol (2,3-BD) producing bacteria with a newly isolated non-pathogenic bacterium, *Raoultella ornithinolytica* B6, which can produce 2,3-BD efficiently without 1,3-propanediol production by using glycerol. The specific objectives of this dissertation were as follows:

1) Isolation and identification of a new species for 2,3-BD production.
2) Characterization of a newly isolated 2,3-BD producing bacterium, *Raoultella ornithinolytica* B6.
3) Optimization of fermentation conditions for improving 2,3-BD production from glucose and glycerol.
4) Improvement of 2,3-BD production by overexpression of the homologous *budABC* genes involved in conversion of pyruvate to 2,3-BD

Chapter 2

Isolation and characterization of a 2,3-butanediol-producing bacterium *Raoultella ornithinolytica* B6 from a soil sample

2.1. Summary

2,3-Butanediol (2,3-BD) is a very useful chemical, which has been used as a solvent, a liquid fuel, and a precursor of many synthetic polymers. Screening of bacteria in a soil sample was carried out to find a new 2,3-BD producer, which can produce 2,3-BD by using glycerol as well as carbohydrate.

The 2,3-BD producing isolate was designated as *Raoultella ornithinolytica* B6 based on 16S rRNA sequence. The B6 strain can produce 2,3-BD as a main product by using various carbon sources including hexoses (glucose, galactose, and fructose), xylose, sucrose, and glycerol. The optimum temperature for growth and 2,3-BD production was 25 °C. Notably, cell growth and 2,3-BD production of *R. ornithinolytica* B6 were higher at 25 °C than at 30 °C.

2.2. Introduction

Bio-chemical production from glycerol has received attention since glycerol price is on the decrease. Glycerol is produced as a by-product in biodiesel production at a weight ratio of 1:10 (glycerol: biodiesel). As biodiesel production increases, the glycerol price is on the decrease (Posada et al., 2012).

Glycerol could be biologically converted to ethanol, 1,3-propanediol (1,3-PD), citric acid, poly(hydroxyalkaoates), and organic acids such as lactic acid, succinic acid, propionic acid, etc. (Luo et al., 2016). 2,3-Butanediol (2,3-BD) can be also produced from glycerol by microorganisms, which used as a solvent and a precursor for many synthetic polymers and chemicals such as methyl ethyl ketone, 1,3-butanedien, 2-butene, etc. These materials are monomers for synthetic rubber and other polymers (Emerson et al., 1982; Gong et al., 1997; Yu & Saddler, 1985).

A few studies about bioconversion of glycerol to 2,3-BD were reported using *K. pneumoniae* (Petrov & Petrova, 2010), *K. oxytoca* (Cho et al., 2015a), *B. amyloliquenfaciens* (Yang et al., 2015), etc. Recently, Ripoll et al. (2016) reported *R. planticola* and *R. terrigena* as biocatalysts for glycerol conversion to 2,3-BD. *R. planticola* and *R. terrigena* have been reclassified from *Klebsiella* genus. It is known that the *Raoultella* species can produce 2,3-BD using glucose (Celinska & Grajek, 2009). 2,3-BD operon and its transcriptional regulator in *R. terrigena* (previously known as *K. terregena*) were characterized (Blomqvist et al., 1993; Mayer et al., 1995). However, the metabolic engineering or fermentation optimization of *Raoultella* species for bio-chemical production has rarely been reported.

This study was focused on isolation and characterization of a 2,3-BD

producing bacterium, which can produce 2,3-BD at high concentration by using glycerol as well as glucose.

2.3. Materials and methods

2.3.1. Media

Enrichment medium contains 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 3 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 0.02 g/L $CaCl_2 \cdot 2\ H_2O$, 0.0092 g/L $FeSO_4 \cdot 7\ H_2O$, 19.52 g/L MES, 1 g/L yeast extract, 30 g/L glycerol, 0.0108 g/L calcium pantothenate, 0.0108 g/L nicotinic acid, 0.2688 g/L myo-inositol, 0.0108 g/L thiamine, 0.0108 g/L pyridoxine, 0.0022 g/L para-aminobenzoic acid, 0.00003 g/L d-biotin, 1mL of SL7 solution containing 1mL/L of 25% HCl, 70 mg/L $ZnCl_2$, 100 mg/L $MnCl_2 \cdot 4H_2O$, 60 mg/L H_3BO_3, 200 mg/L $CoCl_2 \cdot 6\ H_2O$, 20 mg/L $CuCl_2 \cdot 2\ H_2O$, 20mg/L $NiCl_2 \cdot 6\ H_2O$, 40 mg/L $NaMoO_4 \cdot 2\ H_2O$. Fermentation medium (Ji et al., 2008) contained 13.7 g/L K_2HPO_4, 2 g/L KH_2PO_4, 3.3g/L $(NH_4)_2HPO_4$ 6.6g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7\ H_2O$, 0.05 g/L $FeSO_4 \cdot 7\ H_2O$, 0.001 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.001 g/L $MnSO_4 \cdot H_2O$, 0.01g/L $CaCl_2 \cdot 2\ H_2O$, 50 g/L sugar.

2.3.2. Isolation and Identification

A 2,3-BD-producing bacterium was isolated from soil samples of Baegun Mountain in Korea. The soil sample was cultivated in the enrichment medium at 30°C. After enrichment, bacteria in the samples were transferred several times to narrow down the candidate and bacterial metabolites were analyzed by gas-chromatography before subculture. 2,3-BD containing sample was grown on agar plates and single isolated colonies were transferred to 20 ml of the enrichment medium. These procedures were repeated several times to ensure purity of the culture.

For bacterium identification, the genomic DNA (gDNA) was extracted

using the Exgene™ Cell SV mini kit (GeneAll Biotechnology Co.,LTD, Korea). The extracted gDNA was used as a template and the universal primers 27 F and 1492 R were used for the amplification of the 16S rRNA gene. The sequences of the PCR product (1354 bp) were analyzed by Macrogen Inc. (Seoul, Korea) (http://www.macrogen.com). Sequences from the nearest relatives were identified with the Ez-taxon program (Chun et al., 2007).

2.3.3. Flask culture

A single colony from an agar plate was inoculated in the LB medium and incubated in 150 rpm on a shaking incubator overnight at 30°C or 25°C. Then, the seed culture was inoculated with 2% (v/v) in 20ml of fermentation medium with 50 g/L carbohydrates or glycerol. Sampling was carried out periodically to determine pH and concentrations of cell mass, residual substrates and products.

2.3.4. Analytical procedures

Cell growth was determined by measuring the optical density of the culture broth at 600 nm with a spectrophotometer (Shimadzu UV-1240, Japan). The concentrations of sugars and organic acids were analyzed by high performance liquid chromatography (Agilent HPLC, USA) equipped with the Aminex HPX-87 H column ([300×7.8 mm], Bio-rad, USA). The mobile phase (5 mM H_2SO_4), was eluted at a flow rate of 0.6 mL/min at 50 °C. The eluate was monitored with a refractive index detector at 50 °C for sugars and a UV detector at 210 nm for organic acids. The quantifications of fermentation products such as acetoin, 2,3-BD, and ethanol were determined using 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] (USA) under the following conditions: oven temperature, from 50 °C to 240 °C at the rate of 30 °C /min; injector temperature, 240 °C; detector temperature, 250 °C; carrier gas (N_2) flow rate, 30 mL/min.

2.4. Results

2.4.1. Isolation and identification

For isolation of bio-chemical producing bacteria by using glycerol, the oil-contaminated soil sample was collected from Baegun Mountain in Korea.

The isolation procedure from an enriched soil sample under anaerobic condition is shown in Appendix Figure S1. The selection pressures were carbon source (glycerol) and product. From second generation, single colonies (B6.5-1-2-1, B6.5-2-1-1, B6.5-2-1-2, B6.5-3-1-1 and B6.5-3-1-2) were selected, which were most well grown colony at agar plate.

B6.5-3-1-1 produced 7.7 g/L 1,3-PD and B6.5-2-1-1 produced 1.2 g/L butyric acid from glycerol. B6.5-1-2-1 and B6.5-3-1-2 produced ethanol by using glycerol at 5.8 g/L and 3.2 g/L, respectively. Among them, B6.5-1-2-1 produced 5.3 g/L 2,3-BD by using glucose and did not produce 1,3-PD by using glycerol under anaerobic condition. 1,3-PD producer, B6.5-1-1 and butyric acid producer, B6.5-2-1-1 were identified as *Clostridium butylicum* based on 16S rRNA gene sequence with 99.9 % homology. According to comparison of the 16S rRNA gene sequence (Appendix Data S1) of the B6 with those of the related strains, the B6 was identified as *R. ornithinolytica* (JCM6096^{T}) with 99.9% homology. Accordingly, the B6 strain was designated as *R. ornithinolytica* B6 and was deposited as KCCM11176-P in the Korea Culture Center of Microorganisms.

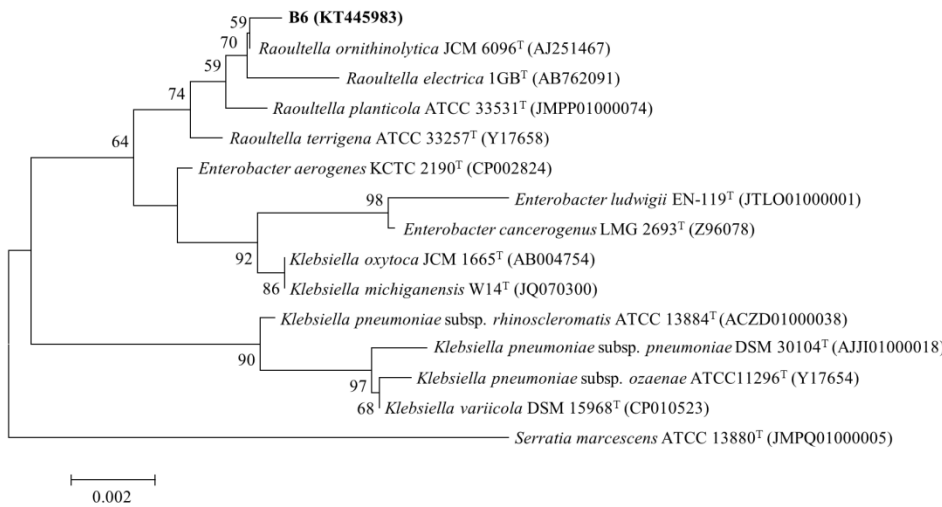

Figure 2. Phylogenetic tree derived from the analysis of the 16S rRNA gene sequences of isolate B6 and related strains. The tree was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bootstrap values are from 1000 replications and only those greater than 50% are shown. *Serratia marcescens* ATCC 13880 was selected as the out group. Evolutionary analyses were conducted in MEGA7. The scale bar indicates 0.2 % nucleotide substitution rate.

2.4.2. Effects of temperature on 2,3-BD production

The bacterium belonging to genus *Raoultella* can grow at relatively low temperatures (Drancourt et al., 2001). The cultivation temperature test for growth and 2,3-BD production was conducted using 50 g/L of glucose or glycerol as a carbon source at 25°C, 30°C (Table 3) and 37°C in a flask. Table 3 shows the effect of temperatures on 2,3-BD production by using glucose. Interestingly, at 37 °C, cell growth was not observed (data not shown). The cell growth, 2,3-BD concentration, and productivity were higher at 25 °C than at 30 °C by 58.7 % , 5.1 %, and 4.5 %, respectively. Moreover, the total organic acid production was much lower at 25 °C compared to that at 30 °C (1.13 vs. 3.34 g/L) (Table 3). This phenomenon is distinguished from other 2,3-BD producing microorganisms such as *K. pneumoniae* and *K. oxytoca* cultivated at 37 °C for 2,3-BD production (Jantama et al., 2015; Jung et al., 2012; Ma et al., 2009).

The effect of temperature on glycerol fermentation of *R. ornithinolytica* B6 was also tested at 25 °C and 30 °C in flasks. As shown in Table 4, 2,3-BD concentration at 25 °C was 16.9 g/L, whereas only 3.6 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 substantial production of ethanol and acetic acid at 30 °C.

Table 3. Effects of culture temperature on 2,3-BD production from glucose

Temperature	Time (hours)	pH	O.D. at 600nm	2,3-BD (g/L)	Acetoin (g/L)	Ethanol (g/L)	Total organic acid[1)] (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
25 °C	36	5.36 ± 0.01	11.49 ± 0.26	14.7 ± 0.3	2.5 ± 1.0	3.2 ± 0.3	1.1 ± 0.8	0.29 ± 0.01	0.41 ± 0.01
30 °C	36	4.97 ± 0.26	7.24 ± 0.05	14.1 ± 0.2	2.1 ± 0.3	3.0 ± 0.3	3.3 ± 0.8	0.30 ± 0.02	0.39 ± 0.01

[1)] Organic acids including acetic acid, succinic acid, and lactic acid

* The data are given as average ± standard deviation of triplicate experiments

Table 4. Effect of culture temperature on the 2,3-BD production from glycerol

Temperature	Time (hours)	pH	O.D. at 600nm	2,3-BD (g/L)	Acetoin (g/L)	Ethanol (g/L)	Acetic acid (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
25 °C	48	5.74 ± 0.03	10.43 ± 0.06	16.9 ± 0.2	1.4 ± 0.2	3.2 ± 0.3	1.4 ± 0.2	0.32 ± 0.01	0.35 ± 0.02
30 °C	49	5.02 ± 0.02	4.21 ± 0.11	3.6 ± 0.1	2.0 ± 0.0	3.5 ± 0.1	5.9 ± 0.0	0.17 ± 0.00	0.07 ± 0.00

* The data are given as average ± standard deviation of triplicate experiments

2.4.3. Sugar utilization

Because lignocellulosic biomass (Unrean, 2016) and marine biomass (Wi et al., 2009) are composed of various sugars and these materials have received attention as carbon sources of microorganisms for producing value-added materials, utilization of various carbon sources is an essential feature of industrial microorganisms. Thus, various sugar substrates (glucose, galactose, fructose, xylose and sucrose) were used to evaluate the carbon source utilization capability of *R. ornithinolytica* B6.

The test was performed during 48 hours and Table 5 shows the data at the point of maximum 2,3-BD concentration. The 2,3-BD titer and productivity were different depending on carbon sources used. All the tested substrates supported the growth of *R. ornithinolytica* B6, and 2,3-BD was produced as a main product with varying levels of by-products. Although the productivity was higher with sucrose (0.68 g/L/h) than with glucose (0.59 g/L/h), the highest 2,3-BD titer (21.3 g/L) and yield (0.34 g/g) were obtained with glucose. Moreover, the concentration of organic acids was lower with glucose.

Table 5. Production of 2,3-BD and other metabolites using various carbon sources at 25 °C (initial carbon source of 50 g/L and pH 7.0)

Carbon sources	Time (hours)	pH	O.D. at 600nm	2,3-BD (g/L)	Acetoin (g/L)	Ethanol (g/L)	Total organic acid [1)] (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
Glucose	36	5.71 ± 0.00	10.72 ± 0.45	21.3 ± 1.2	1.0 ± 0.3	3.3 ± 0.0	0	0.34 ± 0.01	0.59 ± 0.03
Fructose	36	5.59 ± 0.01	10.43 ± 0.86	20.6 ± 1.7	1.0 ± 0.2	3.0 ± 0.0	0.4 ± 0.6	0.31 ± 0.01	0.57 ± 0.05
Galactose	36	5.72 ± 0.03	11.28 ± 0.37	19.7 ± 2.0	1.0 ± 0.1	3.5 ± 0.2	1.1 ± 0.0	0.24 ± 0.03	0.55 ± 0.05
Xylose	48	5.03 ± 0.06	13.61 ± 0.34	18.8 ± 1.9	1.6 ± 0.1	2.5 ± 0.0	3.7 ± 0.0	0.24 ± 0.02	0.39 ± 0.02
Sucrose	24	5.94 ± 0.02	13.94 ± 0.09	16.4 ± 0.4	5.7 ± 0.1	2.4 ± 0.1	4.1 ± 0.9	0.29 ± 0.01	0.68 ± 0.02

[1)] Organic acids including acetic acid, succinic acid, and lactic acid

* The data are given as average ± standard deviation of triplicate experiments

2.5 Discussion

R. ornithinolytica is generally found in aquatic environmental samples, insects, and fishes. Unlike other *Klebsiella* species, human infection by *R. ornithinolytica* is very rare (Sun et al., 2015). In fact, *R. ornithinolytica* B6 did not grow at 37 °C, close to the normal body temperature, which could be a reason for rare infection in humans by *R. ornithinolytica*. Recently, *R. ornithinolytica* has been reported as a possible symbiotic microorganism for earthworms and produces metabolites exhibiting antitumor, antifungal, and anticancer effects (Fiolka et al., 2015; Fiolka et al., 2013). *R. ornithinolytica* was also shown to promote the degradation of microalgal cell wall through cellulolytic activities at low temperatures which resulted in an increase of biogas production (Munoz et al., 2014). In addition to those previous results, 2,3-BD production by *R. ornithinolytica* B6 can be a new application of *Raoultella* species.

R. ornithinolytica B6 can utilize various sugars including glucose, galactose, fructose, xylose and sucrose. Because the biorefinery based on renewable resources composed of different carbon sources has continually attracted attention, the broad substrate spectrum of *R. ornithinolytica* B6 is advantageous in utilizing lignocellulosic biomass for 2,3-BD production. Moreover, the B6 strain could produce 2,3-BD using glycerol, which is a promising renewable resource. The studies on 2,3-BD production using glycerol are less than those using saccharides.

In conclusion, the newly isolated bacterium, *R. ornithinolytica* B6 can be considered as a potential candidate for 2,3-BD production.

Chapter 3

High production of 2,3-butanediol production by newly isolated *Raoultella ornithinolytica* B6 through optimization of fermentation conditions and metabolic engineering

3.1. Summary

Biological production of 2,3-butandiol (2,3-BD) has received great attention as an alternative to the petroleum-based 2,3-BD production. In this study, production of 2,3-BD in fed-batch fermentation was investigated with *Raoultella ornithinolytica* B6. The effects of pH-control schemes, and agitation speed on 2,3-BD production were explored to optimize the fermentation conditions.

To improve 2,3-BD production, the optimization of a fed-batch fermentation condition was performed. At first, the three pH control schemes were tested; no-pH control, constant pH at 7.0, and constant pH at 5.5 after the pH naturally decreased from initial pH 7 called “two-stage pH control”. With applying the two-stage pH control strategy, 2,3-BD production increased by a 1.5-fold compared with no pH-control. Second, to determine the optimum aeration condition for 2,3-BD production of *R. ornithinolytica* B6, the fermentation was conducted with 1vvm airflow under 300, 400, and 500 rpm. The highest concentration of 2,3-BD (68.3 g/L) was produced at 400 rpm. Further improvements of 2,3-BD production in titer (112.2 g/L), yield (0.38 g/g) and productivity (1.35 g/L/h) were achieved by the overexpression of the homologous *budABC* genes, the 2,3-BD producing related genes directly involved in the conversion of pyruvate to 2,3-BD.

3.2. Introduction

2,3-Butanediol (2,3-BD) is a promising platform chemical to replace the petroleum based chemicals. 2,3-BD could be converted to methyl ethyl ketone, 1,3-butanedien, 2-butene, etc (Cho et al., 2013; Ji et al., 2009), which are monomers for synthetic rubber and other polymers (Emerson et al., 1982; Gong et al., 1997; Kim et al., 2014b; Yu & Saddler, 1985).

There are extensive studies on 2,3-BD production by microbial fermentations using carbohydrates through the optimization of fermentation conditions and metabolic engineering. Ma et al. (Ma et al., 2009) reported high production of 2,3-BD (150 g/L) with *K. pneumoniae* SDM through optimization of a feeding strategy. *S. marcescens* H30 (a random mutant of *S. marcescens* A3) and a surfactant-deficient mutant of *S. marcescens* H30 produced 139.9 g/L and 152 g/L 2,3-BD by optimization of the medium composition(Zhang et al., 2010b) and inactivation of the *swrW* gene(Zhang et al., 2010a), respectively. Production of 2,3-BD by *E. aerogenes*, *K. pneumoniae* and *K. oxytoca* was reported to be dramatically increased by deletion of the NADH-requiring by-product synthesis genes (Jantama et al., 2015; Ji et al., 2010; Jung et al., 2012; Park et al., 2013b) and the overexpression of the 2,3-BD synthesis related genes (Cho et al., 2015b; Kim et al., 2014a; Kim et al., 2012). In addition to those microorganisms, well-known model hosts such as *S. cerevisiae* (Kim et al., 2014b) and *E. coli* (Tong et al., 2016) have also been engineered to produce 2,3-BD. Among those 2,3-BD producers, *K. oxytoca* (Cho et al., 2015a) and *K. pneumoniae* (Ma et al., 2009) are considered to be promising producers for 2,3-BD. Other *Klebsiella* species have not been extensively investigated as 2,3-BD producers.

Meanwhile, several *Klebsiella* species including *K. terrigena*, *K. ornithinolytica*, and *K. planticola* have been reclassified to the *Raoultella* genus (Drancourt et al., 2001). *Raoultella* species are Gram-negative, non-motile, aerobic and facultative anaerobic having a respiratory as well as a fermentative metabolism (Drancourt et al., 2001). Unlike *Klebsiella* species which have critical human medical issues, *Raoultella* species are generally isolated from environmental samples, insects, and fishes (Drancourt et al., 2001; Sun et al., 2015). The *Raoultella* species produce 2,3-BD using glucose (Celinska & Grajek, 2009). There are studies about identification and characterization of the genes related to 2,3-BD production in *R. terregina* (Blomqvist et al., 1993; Mayer et al., 1995). However, the use of *Raoultella* species for biological production of chemicals through fermentation as well as metabolic engineering has not been reported to date.

In this study, a newly isolated bacterium, *R. ornithinolytica* B6 was investigated as another promising candidate microbe for high production of 2,3-BD. *R. ornithinolytica* B6 produced 2,3-BD as a main product using hexose, pentose, and sucrose. The optimized fermentation conditions of *R. ornithinolytica* B6 were established by comparing 2,3-BD production at different temperatures, pH control schemes, and agitation speeds. To further increase the carbon flux toward 2,3-BD synthesis and to decrease by-product formation, metabolic engineering was conducted by overexpressing the 2,3-BD synthesis related genes: *budB*, *budA*, and *budC* encoding acetolactate synthase, acetolactate decarboxylase, and acetoin reductase (AR), respectively. The results demonstrated that *R. ornithinolytica* B6 has potential as a high 2,3-BD producer.

3.3. Materials and methods

3.3.1. Microorganism and media

R. ornithinolytica B6 deposited in the Korea Culture Center of Microorganisms (KCCM) as KCCM11176-P was used as the parent strain. Fermentation medium contains 13.7 g/L K_2HPO_4, 2 g/L KH_2PO_4, 3.3g/L $(NH_4)_2HPO_4$ 6.6g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7\ H_2O$, 0.05 g/L $FeSO_4 \cdot 7\ H_2O$, 0.001 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.001 g/L $MnSO_4 \cdot H_2O$, 0.01g/L $CaCl_2 \cdot 2\ H_2O$, 50 g/L sugar. 5 g/L yeast extracts and 10 g/L casamino acids were added as indicated in the text.

3.3.2. Fed-batch fermentation

To find an optimum fermentation condition for 2,3-BD production of *R. ornithinolytica* B6, fed-batch fermentation was performed under various pH condition and agitation speed. At first, the experiments were conducted to find an optimum pH for fed-batch fermentation. Fed-batch culture was performed in a 3-L bioreactor (Fermentec, Korea) with 1-L initial medium. The seed culture was inoculated 10% (v/v) into the fermentation medium with initial pH 7.0. The cultivation was carried out at 25°C, 200 rpm of agitation speed with 0.5 vvm air flow in defined medium and pH was controlled as follows: i) without pH control, ii) maintaining initial pH, iii) maintaining at pH 5.5 after naturally decreasing to 5.5. To control pH, 5N KOH was added automatically. Consequently, the agitation speed effect was investigated under the following condition: 25°C, air flow at 1 vvm, stirring at 300, 400 and 500 rpm. When the residual glucose concentration dropped below 20 g/L, 100 mL of glucose stock solution (600 g/L) was supplied.

3.3.3. Acetoin reductase (AR) activity assay

To estimate the AR activity toward acetoin reduction and 2,3-BD oxidation, a *budC* (encoding AR) overexpression mutant was constructed. Plasmids pUC18CM and pUC18CM-*budC* were constructed as previously reported (Cho et al., 2013). The chloramphenicol resistant gene was amplified from 708-FLPe plasmid (Gene Bridges, Germany). The 829 bp *Aat*II-*Aat*II fragment of the chloramphenicol resistant gene was ligated into pUC18 (GentScript, USA) for the construction of pUC18CM. The primers for the overexpression of *budC* were budC-F (5'-TTTTCTAGAATGCAAAAAGTCGCC-3') and budC-R (5'-TTTAAGCTTTTAGTTAAAAACCATACCG-3'). The PCR product was inserted into the *Xba*I and *Hind*III restriction sites of pUC18CM. *E. coli* HIT-DH5α (RBC Bioscience Corp, Taiwan) was used to clone these genes. The transformation of pUC18CM-*budC* to *R. ornithinolytica* B6 was done by electroporation of the plasmid at 12.5 kV/cm, 200 Ω, and 25 μF with Gene PulserXcell (Bio-rad).

R. ornithinolytica B6 (pUC18CM-*budC*) was cultivated in 20 mL LB medium with 30 μg/mL of chloramphenicol for 12 hours at 25 °C. A harvested cell pellet was suspended in 50 mM potassium phosphate buffer (pH 7.0) and sonicated with VCX 750 (SONICS & MATERIALS, INC., USA). Before measurement of the AR activity, the buffer was exchanged with a fresh buffer using an Amicon ultrafiltration unit with a 10 kDa cut-off (Millipore, Billerica, MA, USA), and crude proteins larger than 10 kDa including AR (27 kDa) were used for the AR activity assay. The activities of AR for acetoin reductase and 2,3-BD oxidation were assayed as previously described (Cho et al., 2015b). Briefly, acetoin reduction activity of AR was determined spectrophotometrically by measuring the consumed

NADH at 340 nm using the molar extinction coefficient of NADH over 5 minutes (Cary 60 UV-Vis, Agilent Technologies, USA). The reaction mixtures containing 50 mM potassium phosphate buffers (pH 7) with 0.15 mM NADH and 1.0 mM acetoin were incubated for 5 minutes at 25°C. After adding crude protein (25 ug/mL) the reaction was started. One unit of AR activity was defined as the amount of crude protein required to reduce 1 μmol of NADH in 1 min. The 2,3-BD oxidation activity assay was at 25 °C and pH 7.0 with 0.15 mM NAD^+ and 1.0 mM 2,3-BD. 2,3-BD oxidation activity of AR was analyzed by measuring the generated NADH in absorbance at 340 nm.

3.3.4. Overexpression of *budABC*

The genes encoding acetolactate decarboxylase (*budA*) (accession number AGJ85611), acetolactate synthase (*budB*) (accession number AGJ85610), and AR (*budC*) (accession number AGJ85609) were derived from the full genome of *R. ornithinolytica* B6. The *budA*, *budB* and *budC* genes consist of the *bud* operon with an amino acid similarity of 96%, 95% and 96% to the corresponding genes of *K. terrigena* (Blomqvist et al., 1993). The primers for the overexpression of *budABC* were budA-F (5'-TTTGAATTCCACAAGACTAAGGAGGCCA CAATGACCCATTCTTCTGC-3') and budC-R (5'-TTTCTCGAGTTAGTTAAAAACCAT ACCG-3'). The PCR product was inserted into the *Eco*RI and *Xho*I restriction sites of pBbA5c-RFP (Addgene, USA) (Kim et al., 2015a), resulted in pBbA5c-*budABC*. The pBbA5c-RFP vector contains p15A origin, IPTG inducible gene expression system with the lac promoter, and chloramphenicol resistance marker. The pBbA5c-RFP contains the restriction enzyme site of, *Eco*RI, *Bgl*II, *BamH*I, and *Xho*I, in sequence. The *rfp*

gene is located between the restriction site of *Bgl*II and *BamH*I. Because cleavage with *Bgl*II and *BamH*I produces compatible overhangs, the empty vector, pBbA5c, was constructed through the digestion of pBbA5c-RFP with *Bgl*II and *BamH*I followed by ligation. The expression of target genes was induced with 0.5 mM IPTG after 3 hours of incubation. MAX Efficiency® DH10B™ (Invitrogen, USA) was used to clone these genes. *R. ornithinolytica* B6 was used as a host strain, and transformation was done by electroporation as described above. *R. ornithinolytica* B6 harboring pBbA5c-*budABC* and pBbA5c was cultured with 30 μg/mL of chloramphenicol to maintain the plasmid.

3.3.5. SDS-PAGE for confirmation of *budABC* overexpression

R. ornithinolytica B6, *R. ornithinolytica* B6 (pBbA5c), *R. ornithinolytica* B6 (pBbA5c-RFP), and *R. ornithinolytica* B6 (pBbA5c-*budABC*) were grown in the medium described in the materials and method section. Except for the wild type, 30 μg/mL of chloramphenicol was added. When OD_{600} was reached at 1.0, 0.5 mM IPTG was added. After 3 hours of induction, the cells were harvested to obtain soluble enzymes using BugBuster Master Mix (Merch, Germany). The enzyme of 10 ug was loaded onto the 10 % SDS-PAGE gel. The electrophoresis was performed using 1/10 diluted 10x Tris/Glycine/SDS buffer (Bio-rad, USA) at 200 V for 40 min. The gel was stained using Coomassie Brilliant Blue R250 staining solution (Bio-rad, USA).

3.3.6. Analytical procedures

Cell growth was determined by measuring the optical density of the culture broth at 600 nm with a spectrophotometer (Shimadzu UV-1240, Japan). The concentrations of sugars and organic acids were analyzed by high performance liquid chromatography (Agilent HPLC, USA) equipped with the Aminex HPX-87 H column ([300×7.8 mm], Bio-rad, USA). The mobile phase was 5 mM H_2SO_4, was eluted at a flow rate of 0.6 mL/min at 50 °C. The eluate was monitored with a refractive index detector at 50 °C for sugars and a UV detector at 210 nm for organic acids. The quantifications of fermentation products such as acetoin, 2,3-BD, and ethanol were determined using 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] (USA) under the following conditions: oven

temperature, from 50 °C to 240 °C at the rate of 30 °C /min; injector temperature, 240 °C; detector temperature, 250 °C; carrier gas (N_2) flow rate, 30 mL/min.

3.4. Results

3.4.1. Effects of pH-control on 2,3-BD production in fed-batch fermentation

In the batch cultures of *R. ornithinolytica* B6, acid formation along with 2,3-BD production generally occurred causing a pH drop. The effect of pH-control on production of 2,3-BD and organic acids in fed-batch fermentation was investigated in defined medium with 0.5 vvm airflow and 200 rpm of agitation speed. Three pH-control schemes with the initial pH at 7 were tested: i) no pH control; ii) pH control at 7, and iii) pH control at 5.5 once the pH decreased to 5.5 during fermentation. When the pH was not controlled, the pH decreases along with cell growth from pH 7 to pH 4.6. Consequently, 136.0 g/L of glucose was consumed to produce 42.7 g/L of 2,3-BD in 96 hours (Figure 3a). Because the low-pH could inhibit the cell growth, pH was controlled at initial pH 7.0 in the followed experiments (Figure 3b). In this case, the cell growth and glucose consumption were increased by 1.3-fold and by 1.4-fold, respectively, compared that when pH was not controlled. However, acids production was stimulated. As a result, the main product was lactic acid (45.8 g/L) not 2,3-BD (35.0 g/L).

Based on the previous experiments, the initial pH was set at 7.0 for securing cell mass in the early stage of fermentation. When the pH reaches 5.5, it was maintained for preventing the acid production (Figure 3c). As a result, *R. ornithinolytica* B6 produced 64.7 g/L 2,3-BD using 202.6 g/L glucose. Moreover, adjusting the pH at 5.5 effectively reduced lactic acid production (3.1 g/L) compared to the fed-batch fermentation at pH 7.

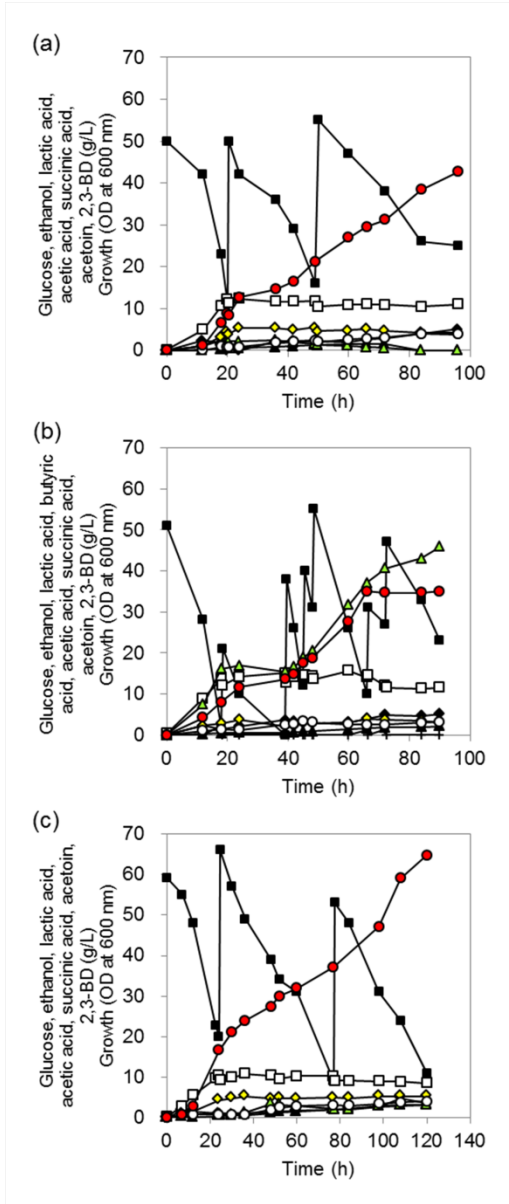

Figure 3. Fed-batch fermentation of glucose by R. ornithinolytica B6 under various pH conditions; (a) without pH control; (b) with pH maintained at 7.0; (c), pH controlled at 5.5 after pH dropped to 5.5 from the initial pH of 7.0. ; ■, glucose; □, growth; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid; ▲, succinic; +, butyric acid..

Considering the previous results by Jung et al. (2012) and Park et al. (2013b) in which the enhancement of 2,3-BD production was obtained by reducing lactic acid formation with lactate dehydrogenase-deleted mutants, the two stage pH control strategy (controlling pH at 5.5 after the pH reached 5.5) attempted here would be a relatively simple and convenient method to reduce lactic acid production. Because 2,3-BD production (64.7 g/L), yield (0.32 g/g), and productivity (0.54 g/L/h) obtained with pH control at 5.5 were higher than those of the other two pH scheme fed-batch fermentations, all further studies were done by maintaining the pH at 5.5 after the pH dropped from 7.0 to 5.5.

3.4.2. Effects of agitation speed on 2,3-BD production in fed-batch fermentation

Aeration is also an important factor for high production of 2,3-BD. Because it is relatively simple, the control of agitation speed is widely used for aeration control instead of regulation of dissolved oxygen concentration (Cho et al., 2015b; Ji et al., 2009; Park et al., 2013b).

The effect of the agitation speed on 2,3-BD production by *R. ornithinolytica* B6 was investigated at agitation speeds of 300 rpm, 400 rpm, and 500 rpm in fed-batch fermentation with 1 vvm airflow. To enhance cell growth and 2,3-BD production, 5 g/L yeast extracts and 10 g/L casamino acids were added to fermentation medium (Cho et al., 2015b; Jung et al., 2012). For the agitation speed controlled at 300 rpm, 2,3-BD was produced up to 54.2 g/L with the productivity of 0.57 g/L/h. But 12.1 g/L acetoin and 14.7 g/L acetic acid were also produced (Figure 4a). When agitation speed was increased to 400 rpm, 2,3-BD production increased up to 68.3 g/L with the productivity of 0.78 g/L/h, the yield of 0.30 g/g (Figure 4b). Moreover, by-products including lactic acid, succinic acid, ethanol and

acetoin decreased significantly compared that at 300 rpm, indicating that 400 rpm was efficient to reduce by-product formation. In contrast, acetic acid, a none NADH-requiring by-product, continuously increased during the fermentation, and acetic acid concentration at 400 rpm was higher than that at 300 rpm by a 1.59 fold. In addition, cell growth was much enhanced at 400 rpm compared to that at 300 rpm. However, with the more increase of agitation speed (500 rpm), cell growth was further enhanced, and acetic acid (18.1 g/L) was the main product with the yield of 0.19 g/g. Only 7.9 g/L 2,3-BD was produced during 105 hours of fermentation (Figure 4c).

These results indicate that a high agitation speed stimulated acetic acid synthesis and cell growth; therefore, the selection of a proper agitation speed is required for the high production of 2,3-BD. Because the highest titer of 2,3-BD production using *R. ornithinolytica* B6 was achieved at 400 rpm, all further fed-batch fermentations were conducted at an agitation speed of 400 rpm.

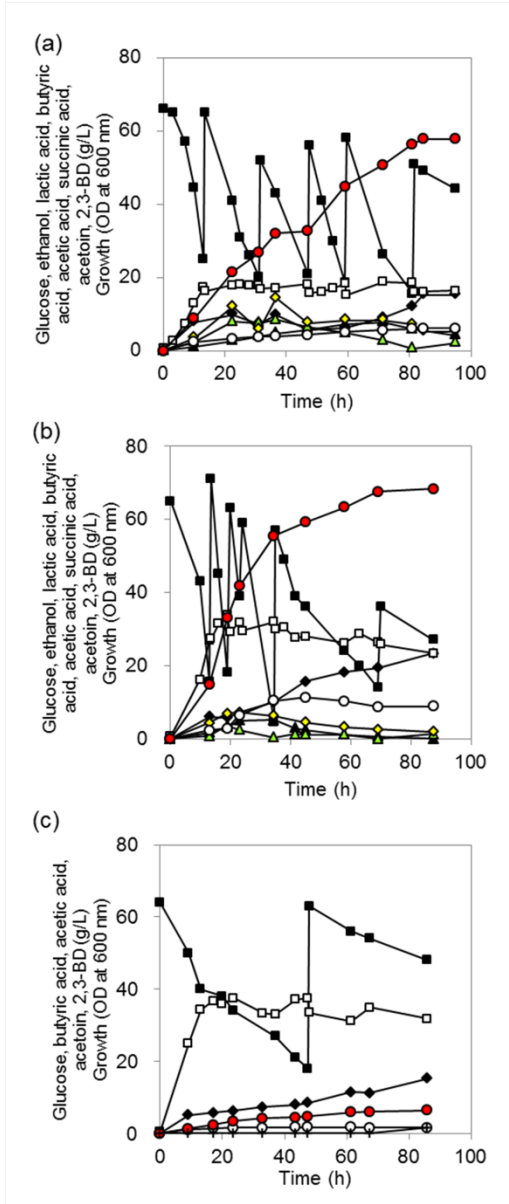

Figure 4. Fed-batch fermentation of glucose by *R. ornithinolytica* B6 at various agitation speeds. pH controlled at 5.5 after the pH reached 5.5 from an initial pH of 7.0 under several agitation speeds (a) 300 rpm, (b) 400 rpm, (c) 500 rpm; ■, glucose; □, growth; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid; ▲, succinic; +, butyric acid.

3.4.3. Overexpression of *budA*, *budB* and *budC* genes

Further improvement of 2,3-BD production was attempted by overexpressing the 2,3-BD synthesis-related genes including acetolactate synthase (encoded by *budB*), acetolactate decarboxylase (encoded by *budA*), and 2,3-butanediol dehydrogenase (encoded by *budC*).

In the fed-batch fermentation with *R. ornithinolytica* B6, although much less NADH-requiring metabolites (e.g., lactic acid, succinic acid, and ethanol) were produced at 400 rpm than at 300 rpm, acetic acid production at 400 rpm was significantly increased compared to that at 300 rpm. Because the partitioning of carbon flux at the pyruvate node to acetic acid (via acetyl Co-A) and 2,3-BD (via α-acetolactate) occurs, the *budABC* genes were overexpressed to increase the carbon flux to 2,3-BD and consequently to decrease the carbon flux to acetic acid. The overexpression of *budABC* in *R. ornithinolytica* B6 (pBbA5c-*budABC*) was confirmed by SDS-PAGE as shown in Figure 5.

Finally, using *R. ornithinolytica* B6 (pBbA5c-*budABC*), 2,3-BD concentration was dramatically increased to 112.2 g/L (Figure 7) with a yield of 0.38 g/g and a productivity of 1.35 g/L/h which were higher than those of *R. ornithinolytica* B6 (pBbA5c) (i.e., the control, Figure 6) by 1.84-fold, 1.23-fold and 1.88-fold, respectively. Acetic acid production by *R. ornithinolytica* B6 (pBbA5c-*budABC*) was significantly reduced to 9.7 g/L compared to that of the control, indicating a decrease of the carbon flux at the pyruvate node to acetic acid. In contrast, *R. ornithinolytica* B6 harboring pBbA5c-*budABC* produced more acetoin than that of the control, indicating that acetoin conversion to 2,3-BD was somewhat limited. Among the 2,3-BD synthesis-related genes, AR is known to

catalyze both the reduction of acetoin to 2,3-BD and the reverse reaction (the oxidation of 2,3-BD to acetoin) (Syu, 2001; Yang et al., 2014). Therefore, the activity of AR toward 2,3-BD synthesis was compared to that of the reverse reaction. When the crude protein extract from *R. ornithinolytica* B6 (pUC18CM-*budC*) was used to measure the AR activity, the AR activity toward acetoin reduction to 2,3-BD was 10-fold higher than that of the reverse activity (3.21 vs. 0.32 U/mg protein), implying that the oxidation of 2,3-BD to acetoin by AR would not be the reason for high acetoin concentration.

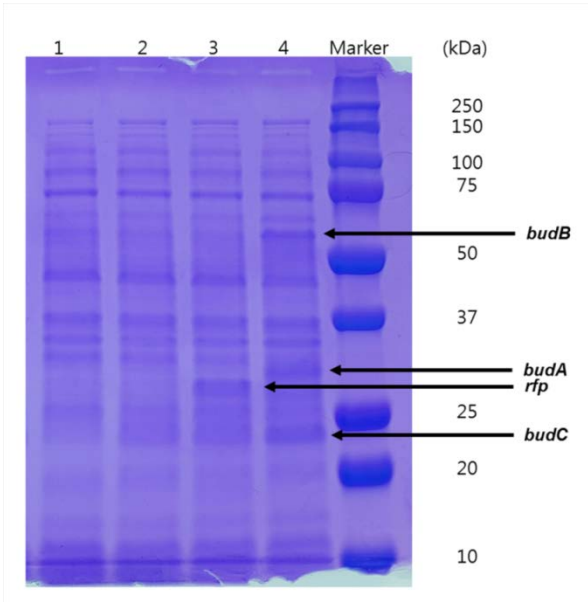

Figure 5. SDS-PAGE analysis of gene overexpression in the *R. ornithinolytica* B6 (pBbA5c-*budABC*) comparing with wild-type strain. The *rfp* (28 kDa), *budA*(29 kDa), *budB* (61 kDa) and *budC* (26 kDa) bands are indicated by arrows. The samples were prepared after 3 hours of induction with 0.5 mM IPTG (lanes 2,3,4); lane 1, *R.ornithinolytica* B6; lane 2, *R.ornithinolytica* B6 (pBbA5c); lane 3, *R.ornithinolytica* B6 (pBbA5c-RFP); lane 4, *R.ornithinolytica* B6 (pBbA5c-*budABC*).

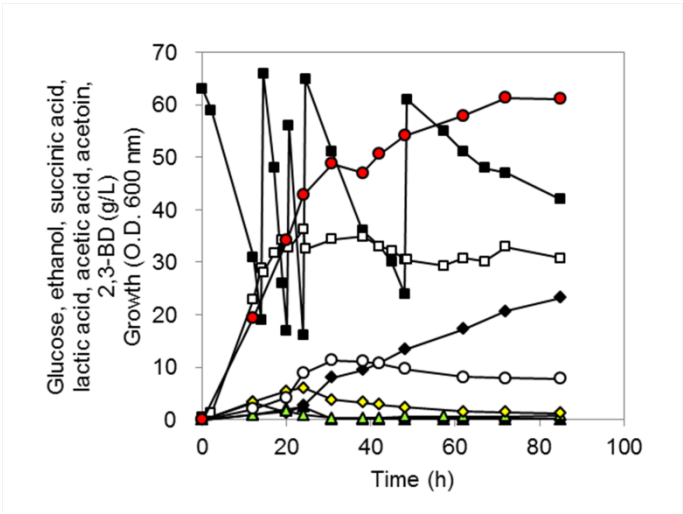

Figure 6. Fed-batch fermentation of glucose by *R. ornithinolytica* B6 (pBbA5c); ■, glucose; □, growth; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid; ▲, succinic acid.

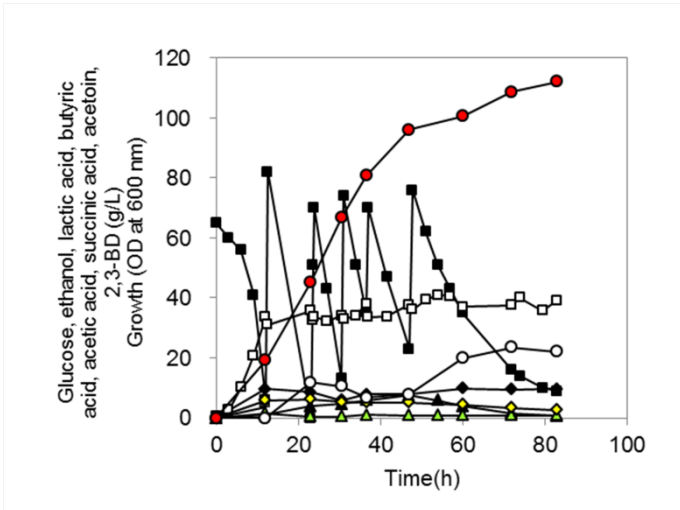

Figure 7. Fed-batch fermentation of glucose by *R. ornithinolytica* B6 (pBbA5c-*budABC*); ■, glucose; □, growth; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; △, lactic acid; ▲, succinic; +, butyric acid.

3.5. Discussion

This study is the first report of 2,3-BD production through fed-batch fermentation and metabolic engineering with the *Raoultella* genus.

The two operation schemes were employed for improving 2,3-BD production. One was the two stage pH control strategy. The initial pH was set at pH 7.0 for cell mass production in the early stage of fermentation, and then, pH was maintained at 5.5 after pH naturally decreased to 5.5 by cell growth. In the case of the two stage pH control stage process, *R. ornithinolytica* B6 produced a higher 2,3-BD yield than that of the other pH control schemes (no pH control and pH control at 7). These results corresponded to the previously report that the optimum pH range for 2,3-BD production was pH 5.5-6.5 (Zeng et al., 1990).

The other strategy was the optimization of the agitation speed which has been reported to be critical for high 2,3-BD production (Cho et al., 2015b; Ji et al., 2009; Park et al., 2013b). Fed-batch fermentations at different agitation speeds were conducted to find the optimum oxygen supply for 2,3-BD production by *R. ornithinolytica* B6. As a result, the increased oxygen supply by controlling the agitation speed at 400 rpm improved not only the 2,3-BD concentration and productivity but also the 2,3-BD yield. This result is an advantageous and distinguished 2,3-BD production characteristic of *R. ornithinolytica* compared to the previous reports with other 2,3-BD producers: a high oxygen supply resulted in a decrease in the 2,3-BD yield despite of the increase in the 2,3-BD titer, productivity, and cell growth (Cho et al., 2015b; Ji et al., 2009; Park et al., 2013b).

After optimizing the operation conditions, a metabolically engineered *R. ornithinolytica* B6 strain was constructed by overexpressing of the *budABC* to

improve 2,3-BD production further. The genes are involved in the conversion of pyruvate to 2,3-BD. The enhancement of 2,3-BD production as well as a decrease in acetic acid formation by *R. ornithinolytica* B6 (pBbA5c-*budABC*) was remarkable compared to the results of the wild type (Table 3), showing a substantial increase of carbon flux to the 2,3-BD synthesis pathway at the pyruvate node. Consequently, the yield of 2,3-BD by *R. ornithinolytica* B6 (pBbA5c-*budABC*) was significantly improved compared to the yield with the control strain, *R. ornithinolytica* B6 (pBbA5c) (0.38 vs. 0.31 g/g). Unexpectedly, acetoin accumulated up to 22.0 g/L. In previous reports, high 2,3-BD production along with lower acetoin accumulation was attempted using a two-stage agitation speed control strategy in which the agitation speed was changed from a high rpm to a lower rpm (Ji et al., 2009; Park et al., 2013b). When a two-agitation speed strategy was applied for *R. ornithinolytica* B6 (pBbA5c-*budABC*) fermentation by changing the agitation speed from 400 rpm to 300 rpm after 50 hours of fermentation, the acetoin concentration was reduced to 9.1 g/L; however, the 2,3-BD titer and productivity were also reduced to 101.1 g/L and 1.26 g/L/h, respectively. Future studies might be required to increase NADH availability or AR activity to reduce acetoin accumulation.

In all fed-batch fermentations using *R. ornithinolytica* B6, meso-2,3-BD was dominantly produced (optical purity > 85 %) among (2S,3S)-2,3-BD, (2R,3R)-2,3-BD and meso-2,3-BD. Because most of the industrial application of 2,3-BD are achieved using by meso-2,3-BD (Qiu et al., 2016), the meso-2,3-BD production with high optical purity could be an advantage of *R. ornithinolytica* B6.

In this study, high production of 2,3-BD with *R. ornithinolytica* B6 was

conducted through the optimization of the fermentation conditions and metabolic engineering. *R. ornithinolytica* B6 (pBbA5c-*budABC*) produced 2,3-BD up to 112.2 g/L by increasing the carbon flux to 2,3-BD. This report presents high 2,3-BD production by *R. ornithinolytica* strains, extending a microbial spectrum of 2,3-BD producers over previously known 2,3-BD producers. Comparing this result with previously reported 2,3-BD production by *K. oxytoca* (95.5~ 142.5 g/L) (Cho et al., 2015b; Jantama et al., 2015; Ji et al., 2009; Ji et al., 2010; Park et al., 2013b), *K. pneumoniae* (90~ 150 g/L) (Kim et al., 2014a; Kim et al., 2012; Ma et al., 2009), *S. marcescens* (87.8~ 152 g/L) (Shi et al., 2014; Zhang et al., 2010a; Zhang et al., 2010b), and *E. aerogenes* (118.1 g/L) (Jung et al., 2012), *R. ornithinolytica* B6 can be considered as a potential candidate for high 2,3-BD production with expectation of enhanced 2,3-BD production through further metabolic engineering and process optimization.

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Chapter 4

Efficient production of 2,3-butanediol from glycerol by 1,3-propanediol synthesis-deficient *Raoultella ornithinolytica* B6

4.1. Summary

Crude glycerol is a promising renewable resource for the production of value-added chemicals by biological processes. In 2,3-butanediol (2,3-BD) production from glycerol fermentation, 1,3-propanediol (1,3-PD) is a problematic by-product because its similar physicochemical characteristics to 2,3-BD cause difficulty in downstream processes. In this study, 2,3-BD production without 1,3-PD accumulation was investigated using *R. ornithinolytica* B6, which is deficient in the 1,3-PD synthesis pathway. By verifying the optimum temperature, agitation speed, and pH control strategy, the optimal fermentation conditions for 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 fluctuation which positively affected 2,3-BD production was generated by simply controlling the lower pH limit at 5.5. In fed-batch fermentation under optimum conditions, *R. ornithinolytica* B6 produced 79.3 g/L 2,3-BD, and a further enhancement of 2,3-BD production (89.5 g/L) was achieved by overexpressing the homologous *budABC* genes, which are directly involved in conversion of pyruvate to 2,3-BD. Moreover, *R. ornithinolytica* B6 harboring pUC18CM-*budABC* produced 78.1 g/L 2,3-BD with the yield of 0.42 g/g and the productivity of 0.62 g/L/h using pretreated crude glycerol, which were the highest values for 2,3-BD production from glycerol among 2,3-BD producers without the 1,3-PD production. Considering the highest performance of 2,3-BD production, it is certain that *R. ornithinolytica* B6 is a promising 2,3-BD producer from glycerol.

4.2. Introduction

2,3-Butanediol (2,3-BD) is a value-added bio-based chemical with diverse industrial applications. Many bacteria have been reported to produce 2,3-butanediol through sugar fermentation including *B. amyloliquefaciens* (Yang et al., 2012b), *S. marcescens* (Zhang et al., 2010b), *E. aerugenes* (Jung et al., 2012), *K. pneumoniae* (Ma et al., 2009; Tsvetanova et al., 2014), and *K. oxytoca* (Cho et al., 2015b; Ji et al., 2009; Kim et al., 2013a).

Alternatively, 2,3-BD can be produced from glycerol, which is a renewable resource generated from bio-diesel manufacturing processes as a by-product. Crude glycerol is generated at a weight ratio of 1:10 (glycerol: biodiesel). As bio-diesel production increases, the glycerol price is on the decrease about a 10-fold (Posada et al., 2012). Consequently, glycerol utilization has gained much attention for 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 & Gonzalez, 2013; Feng et al., 2014; Luo et al., 2016).

K. pneumoniae and *K. oxytoca*, which are considered as superior 2,3-BD producers, produced 1,3-PD as a by-product when glycerol was supplied as a sole carbon source (Cho et al., 2015a; Petrov & Petrova, 2010; Petrov & Petrova, 2009). Because glycerol is a more reduced carbon source than glucose, 1,3-PD formation involve in net NADH consumption is usually observed in glycerol fermentation in order to maintain redox balance (i.e., NADH/NAD balance). 1,3-PD can be used as a monomer for polytrimethylene terephthalate used in the fiber and textile industries (Clomburg & Gonzalez, 2013); however, 1,3-PD as a by-product is

impediment in downstream processes of 2,3-BD production because of its similar physicochemical properties to 2,3-BD (Xiu & Zeng, 2008; Zhang et al., 2012). Petrov and Petrova (2010) developed a forced pH fluctuation strategy for improving 2,3-BD production from glycerol fermentation. As a result, 2,3-BD was produced up to 70 g/L but the ratio of 1,3-PD: 2,3-BD (about 0.2:1) did not decrease. Cho et al. (2015a) reported the highest glycerol-based 2,3-BD titers from pure glycerol at 115.0 g/L and from crude glycerol at 131.5 g/L by constructing the *pduC* (encoding glycerol dehydratase) and *ldhA* (encoding lactate dehydrogenase) double deleted mutant of *K. oxytoca* to block the formation of 1,3-PD and lactic acid, respectively. Nevertheless, 1,3-PD production is not blocked completely.

Unlike *Klebsiella* species, *B. amyloliquefaciens*, *R. planticola*, and *R. 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 *K. planticola and K. 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-synthesis 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 & 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.

Previously, *R. ornithinolytica* B6 was reported as another candidate for an industrial 2,3-BD producer. The B6 strain has shown potential as a high 2,3-BD producer with glucose (Kim et al., 2016). According to the complete genome sequence (GenBank accession number CP004142), the 1,3-PD synthesis pathway does not exist in *R. ornithinolytica* B6. In this study, based on previous findings, 1,3-PD-free 2,3-BD production was investigated with *R. ornithinolytica* B6 using pure glycerol and crude glycerol. To further increase 2,3-BD production, metabolic engineering of *R. ornithinolytica* B6 was conducted to increase the carbon flux toward 2,3-BD synthesis by overexpressing the homologous *budABC* genes involved in the conversion of pyruvate to 2,3-BD (Kim et al., 2016).

In this study, high 1,3-PD-free 2,3-BD production was achieved from glycerol with the wild type *R. ornithinolytica* strain as well as an engineered *Raoultella* strain. Furthermore, the values obtained using glycerol as a sole carbon source for the 2,3-BD titer (89.5 g/L), productivity (0.75 g/L/h), and yield (0.41 g/g) are the highest values among 1,3-PD synthesis-deficient 2,3-BD producers.

4.3. Materials and methods

4.3.1. Microorganism and media

R. ornithinolytica B6 deposited in the Korea Culture Center of Microorganisms (KCCM) as KCCM11176-P was used as the parent strain. Fermentation medium contains 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 $MgSO_4 \cdot 7\ H_2O$, 0.05 g/L $FeSO_4 \cdot 7\ H_2O$, 0.001 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.001 g/L $MnSO_4 \cdot H_2O$, 0.01g/L $CaCl_2 \cdot 2\ H_2O$, and 50 g/L pure glycerol or crude glycerol as needed. 5 g/L yeast extracts and 10 g/L casamino acids were added as indicated in the text.

4.3.2. Pretreatment of crude glycerol

Crude glycerol, denoted as AK, was obtained from Aekyung Ind. Korea which manufactures biodiesel with an alkali-catalyzed trans-esterification process. The components of the crude glycerol AK and pretreated crude glycerol AK-T are shown in Table 6. The pretreatment of crude glycerol was conducted according to method of Chi et al. (2007) and 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 glycerol; (2) the pH was adjusted to pH 1-2 with 5 N HCl to precipitation of free fatty acids, which is existed as salt as salt form; (3) the crude glycerol solution is centrifuged at 6000 rpm for 20 minutes; (4) the lower phase was gained and autoclaved.

Table 6. Composition of AK and AK-T

Property[1]	Method	Glycerol	
		AK	AK-T
Glycerol content, wt. %	KSM 1979	68.3	70.23
Water content, wt. %	ASTM E203	4.4	26.605
MONG[2], wt. %	ISO 2464-1973	26.6	2
Ash, wt. %	ASTM ED482	0.7	1.167
Methanol[3], wt, %	GC SGS KR 015 (In-House)	2.8	2.66
Sodium[3], mg/kg	IP 501	10137.5	9107
Magnesium[3], mg/kg	IP 501	18.8	12
Potassium[3], mg/kg	IP 501	108.8	1410

[1]Analyzed by SGS Testing, Korea

[2]MONG (matter organic non-glycerol) was determined by 100 wt, % minus sum of glycerol, water and ash.

[3]For more detail information about crude glycerol, quantity of each element was analyzed independently.

4.3.3. Flask culture

A single colony from an agar plate was inoculated in defined fermentation medium with 30 g/L glycerol and incubated in 200 rpm on a shaking incubator for overnight at 25°C. Then, the seed culture was inoculated with 2% (v/v) into 20 ml of fermentation medium with 50 g/L glycerol in a 100 mL Erlenmeyer flask.

4.3.4. Fed-batch fermentation

To optimize glycerol fermentation condition of *R.ornithinolytica* B6, the agitation speed, medium composition and pH control condition were tested in fed-batch fermentation. The experiments were conducted in a 3-L bioreactor (Fermentec, Korea) with 1-L initial medium. The seed culture (100 mL) was inoculated 10% (v/v) into the fermentation medium with initial pH 7.0. The cultivation was carried out at 25°C. When the residual glycerol concentration dropped below 20 g/L, 100 mL of glycerol stock solution (540 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 N KOH. Finally, the effect of complex nitrogen sources (yeast extract and casamino acid) was tested at the optimum agitation speed and pH control mode.

4.3.5. Overexpression of *budABC*

To improve 2,3-BD production, the plasmid pUC18CM-*budABC* was constructed. The primers and vectors for overexpression are listed Table 7. The *budABC* genes encoding), α-acetolactate decarboxylase (BudA) (accession number AGJ85611), α-acetolactate synthase (BudB) (accession number AGJ85610), and acetoin reductase (BudC) (accession number AGJ85609) 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 *Hind*III restriction sites of pUC18CM. *E. 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.

Table 7. 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[1, 2]		
budA-F	5'- TTTTCTAGAATGACCCATTCTTCTGC -3' (*Xba*I)	This study
budC-R	5'- TTTAAGCTTTTAGTTAAAAACCATACCG -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'- CAAAAGGGAAAGGACGA*GAAGTTCCTATACTTTCTAG* -3'	This study
ldhA-del-F	5'- TATATACCCGCCAGGCCAAAC -3'	This study
ldhA-del-R	5'- GATAACAGCATTCCTGCCGC -3'	This study

[1]Underlined sequences are restriction enzyme sites

[2]Italic sequences are homologous with the FRT region

4.3.6. Deletion of *ldhA* gene

For the observation of influence of lactic acid production on 2,3-BD production, deletion of *ldhA* gene (Accession number WP_004861243) was conducted by λ Red recombination method as previously reported by Cho et al. (2015a). The plasmids and primers are listed Table 7; ldhA-UF and ldhA-UR were used for amplification of upstream regions of *ldhA*. ldhA-DF and ldhA-DR were used for amplification of downstream regions of *ldhA*. Additional PCR using pTOT-FCF plasmid as a template was performed with the primers ldhA-FU-F and ldhA-FD-R. These PCR products were amplified by the primers ldhA-UP and ldhA-DR by the overlap extension PCR method. The PCR products were transformed to *R. ornithinolytica* B6 harboring pRedET (Gene Brideges, 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 (1,150 bp) obtained using the ldhA-del-F and ldhA-del-R primers. To remove the chloramphenicol resistant gene from the chromosome, 707-FLPe (Gene Brideges, Germany) was transformed into the cells and the tetracycline-resistant transformants were selected at 30 °C. The 707-FLPe was cured by cultivation at 37 °C. After removal of chloramphenicol resistant gene, the resulting mutant was confirmed by PCR (300 bp).

4.3.7. Analytical procedures

Cell growth was determined by measuring the optical density of the culture broth at 600 nm with a spectrophotometer (Shimadzu UV-1240, Japan). The concentrations of glycerol, succinic acid and lactic acid were analyzed by high performance liquid chromatography (Agilent HPLC, USA) equipped with the Aminex HPX-87 H column ([300×7.8 mm], Bio-rad, USA). The mobile phase was 5 mM H_2SO_4, was eluted at a flow rate of 0.6 mL/min at 50 °C. The eluate was monitored with a refractive index detector at 50 °C for sugars and a UV detector at 210 nm for organic acids. The quantifications of fermentation products such as acetoin, 2,3-BD, and ethanol were determined using 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] (USA) under the following conditions: oven temperature, from 50 °C to 240 °C at the rate of 30 °C /min; injector temperature, 240 °C; detector temperature, 250 °C; carrier gas (N_2) flow rate, 30 mL/min.

4.4. Results

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

R. ornithinolytica B6 is able to produce 2,3-BD not only from various sugars (glucose, fructose, galactose, xylose, and sucrose) (Kim et al., 2016) but also from glycerol as a sole carbon source in aerobic conditions. 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.

4.4.2. Effect of agitation speed on 2,3-BD production

Because optimizing the agitation speed is very important for high production of 2,3-BD (Ji et al., 2009; Park et al., 2013b), the aeration effect on glycerol fermentation was tested in fed-batch fermentation with four different agitation speeds: 200, 300, 400, and 500 rpm with 1.0 vvm air flow and without pH control (Figure 8 and Table 8). The 2,3-BD concentration and yield at 300 rpm (23.3 g/L, 0.34 g/g) were similar to those at 200 rpm (23.2 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) (Figure 8a and 8b). When the fed-batch fermentation was conducted at 400 rpm, the concentration, yield, and productivity of 2,3-BD increased up to 36.4 g/L, 0.39 g/g, and 0.96 g/L/h, respectively (Figure. 8c). At 500 rpm, 2,3-BD

production was 7.3 g/L only despite of high cell growth (Figure. 8d). Because 2,3-BD production at 400 rpm showed the highest concentration, yield, and productivity, an agitation speed of 400 rpm was selected for further studies.

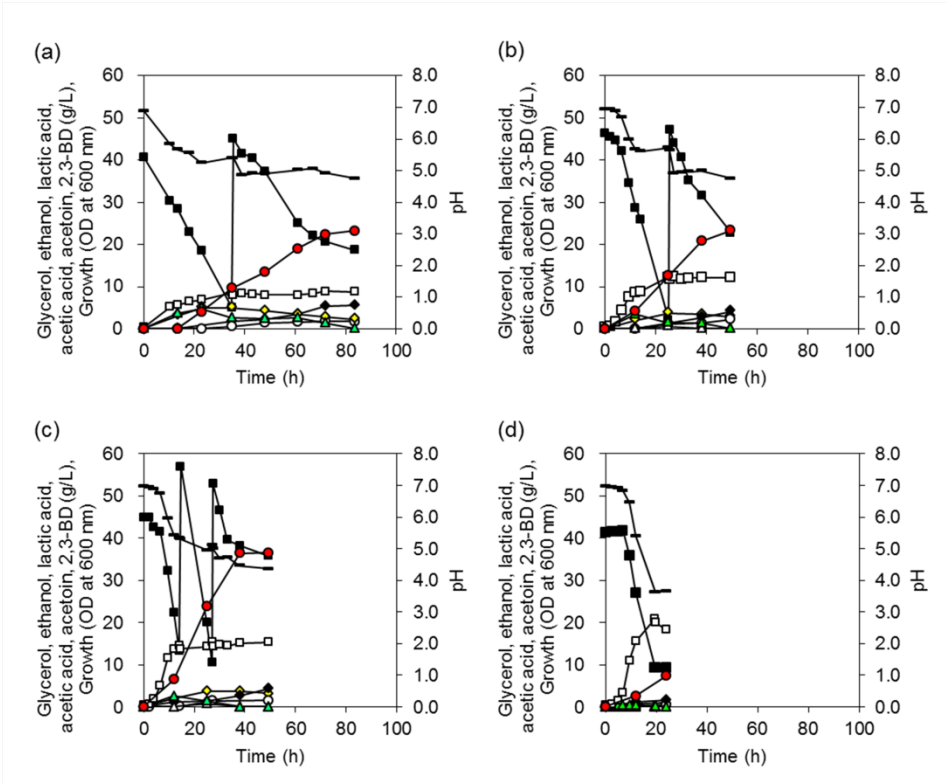

Figure 8. Fed-batch fermentation of glycerol by *R. ornithinolytica* B6 at 25 ˚C in the defined medium without pH control at various agitation speeds (a) 200 rpm, (b) 300 rpm, (c) 400 rpm, (d) 500 rpm; ■, glycerol; □, growth; –, pH; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid.

Table 8. Products in fed-batch fermentation from *R. ornithinolytica* B6 with different agitation speeds

Agitation speeds (rpm)	200	300	400	500
Fermentation time (h)	83.5	49.5	38	24
Consumed substrate (g/L)	66.4	68.6	93.3	38.3
O.D. at 600 nm	8.79 [8.89] [1]	12.13 [12.41]	15.08 [15.38]	18.29 [20.93]
2,3-BD (g/L)	23.2	23.3	36.4	7.3
Acetoin (g/L)	1.8	2.3	1.5	0.5
Acetic acid (g/L)	5.5	4.2	2.7	1.6
Lactic acid (g/L)	0 [2.6]	0 [3.5]	0 [2.6]	0 [0.5]
Succinic acid (g/L)	0 [0.6]	0 [0.6]	0 [0.7]	0
Ethanol (g/L)	2.3	2.9	3.7	0.9
2,3-BD yield (g/g)	0.35	0.34	0.39	0.19
2,3-BD productivity (g/L/h)	0.28	0.47	0.96	0.30

[1] Values in [ ] represent the maximum value for each measurement during the fermentation.

4.4.3. Effect of pH control on 2,3-BD production

In addition to the aeration strategy, another important parameter in 2,3-BD production from glycerol is the pH profiles with fermentation time. Petrov and Petrova (2009) observed that pH fluctuations during effective 2,3-BD production from glycerol (with no pH control mode) were caused by consecutive 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) was effective in enhancing 2,3-BD production up to 70 g/L.

In Figure 8c, when pH was not controlled, the pH profile marginally fluctuated, and 2,3-BD production ceased after 38 hours of fermentation likely due to a significant pH drop to 4.5. To achieve higher 2,3-BD production by preventing acidic conditions while enabling pH fluctuations, 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 hours of fermentation (Figure 9a), an alkali solution (5 N 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 (Figure 9a). With the lower limit of pH 5.5, the pH increased up to 6.0~6.3, and then, 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 (Figure. 9b and 9c). As shown in

Table 9, with the pH control above pH 5.5, 2,3-BD at 60.7 g/L was produced using 154.3 g/L glycerol 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 62.4 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 (Figure. 9d - 9f), 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

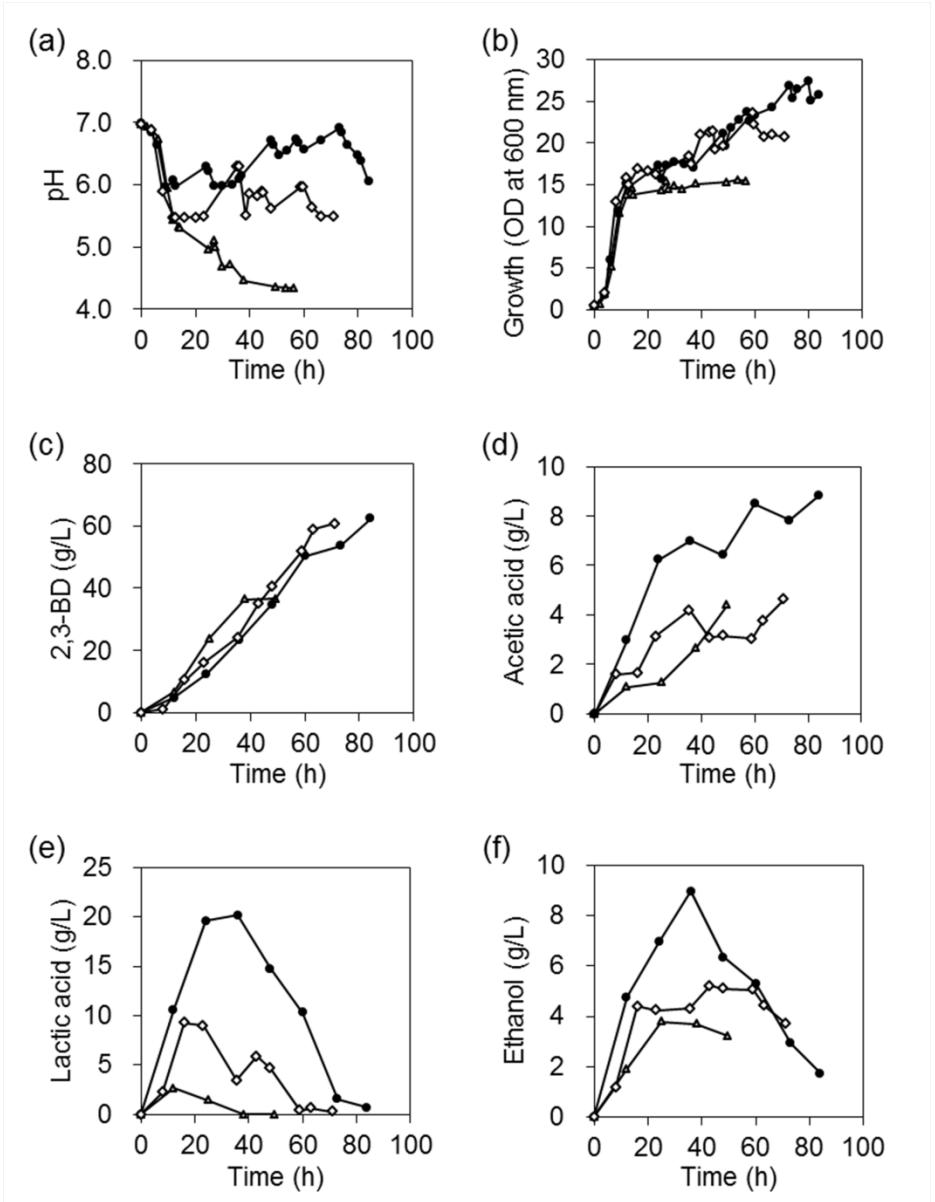

Figure 9. Time course of (a) pH, (b) cell growth, (c) 2,3-BD, (d) acetic acid, (e) lactic acid , (f) ethanol under different pH control modes. Fed-batch fermentation was carried with *R. ornithinolytica* B6 at 25 ˚C and 400 rpm with the defined medium. △, no pH controll; ◊ pH control with the lower limit of 5.5; ●, pH control with the lower limit of 6.0.

Table 9. Products in fed-batch fermentation from *R. ornithinolytica* B6 with different lower pH limits

Lower pH limit	5.5	6.0
Fermentation time (h)	71	84
Consumed substrate (g/L)	154.3	173.4
O.D. at 600 nm	20.79 [23.60] [1]	25.80 [27.42]
2,3-BD (g/L)	60.7	62.4
Acetoin (g/L)	5.7	3.9
Acetic acid (g/L)	4.6	8.8
Lactic acid (g/L)	0.3 [9.3]	0.7 [20.2]
Succinic acid (g/L)	0	0 [0.8]
Ethanol (g/L)	3.7 [5.2]	1.7 [9.0]
2,3-BD yield (g/g)	0.39	0.36
2,3-BD productivity (g/L/h)	0.85	0.74

[1]Values in [ ] represent the maximum value for each measurement during the fermentation.

4.4.4. Effect of complex nitrogen sources on 2,3-BD production

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 Figure 10, 2,3-BD production was significantly enhanced up to 79.3 g/L compared to 2,3-BD titer obtained with the defined medium (60.7 g/L). The addition of complex nitrogen sources appeared to be effective in prolonging the fermentation time up to 123 hours. 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 prolonged fermentation time, the maximum productivities in the middle of fermentation were similar to the defined medium and the complex medium (1.01~1.03 g/L/h) (Table 10).

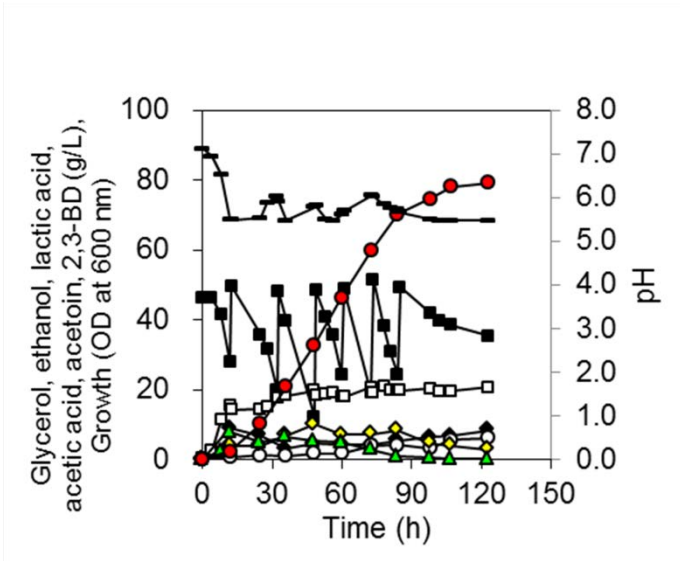

Figure 10. Fed-batch fermentation of glycerol by *R. ornithinolytica* B6 in the complex medium containing 5 g/L yeast extract and 10 g/L casamino acid at 25 ˚C and 400 rpm with the lower pH limit of 5.5; ■, glycerol; □, growth; –, pH; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid.

Table 10. Comparison of products profile in fed-batch fermentation from *R. ornithinolytica* B6 and *R. ornithinolytica* B6 (pUC18CM-*budABC*)

	Strain			
	R. ornithinolytica B6	*R. ornithinolytica* B6 (pUC18CM-*budABC*)		
Glycerol type	pure	pure	AK[2]	AK-T
Fermentation time (h)	123	120	86.5	126
Consumed substrate (g/L)	198.1	218.2	168.5	186.0
O.D. at 600 nm	20.3 [20.9] [1]	34.9 [35.8]	-	24.7 [30.2]
2,3-BD (g/L)	79.3	89.5	65.4	78.1
Acetoin (g/L)	6.2	9.1	11.5	8.8
Acetic acid (g/L)	8.4	1.0	1.2 [2.2]	1.2
Lactic acid (g/L)	0 [7.8]	0 [6.2]	0 [0.6]	0.4 [8.4]
Succinic acid (g/L)	0 [1.0]	0 [1.1]	0 [0.6]	0.1 [1.1]
Ethanol (g/L)	2.84 [9.9]	1.06 [6.5]	0 [9.2]	1.8 [6.5]
2,3-BD yield (g/g)	0.40	0.41	0.39	0.42
2,3-BD productivity (g/L/h)	0.64	0.75	0.76	0.62

[1] Values in [ ] represent the maximum value for each measurement during the fermentation.
[2] Cell growth could not be measured due to the turbidity of crude glycerol, AK.

4.4.5. Correlation between lactic acid production and 2,3-BD production

Even though the 2,3-BD titer was increased by adding complex nitrogen sources, the production of lactic acid was up to 7.8 g/L after 12 hours, and acetic acid production was increased to 8.4 g/L at the end of fermentation (Figure 10).

The production of lactic acid and ethanol is a commonly known problem in mixed-acid fermentations (Petrov & Petrova, 2010) and 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, the 2,3-BD titer (41.9 g/L), yield (0.27 g/g) and productivity (0.59 g/L/h) all were decreased. Meanwhile ethanol concentration increased up to 22.3 g/L at 40 hours (figure 11a). This result implies that the increased carbon and redox power in the *ldhA* deletion mutant was likely utilized for 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.

To decrease ethanol production and to increase 2,3-BD production, *R. ornithinolytica* B6 *Δldh* (pUC18CM-*budABC*) was constructed. As expected, the 2,3-BD production was increased and ethanol production was decreased but the titer of 2,3-BD was still lower than that of the wild type. 56.3 g/L 2,3-BD was produced with 0.35 g/g yield and 0.54 g/L/h of productivity in fed-batch fermentation using *R. ornithinolytica* B6 *Δldh* (pUC18CM-*budABC*) (figure 11b).

The results demonstrate that the pH fluctuation caused by lactic acid formation exerted a crucial role in the 2,3-BD production from glycerol fermentation. Considering the production of lactic acid, ethanol, and 2,3-BD needs NADH, the lactic acid formation in the early stage of fermentation and re-assimilation in the exponential stage of fermentation seems closely related to distribution of NADH in the whole fermentation.

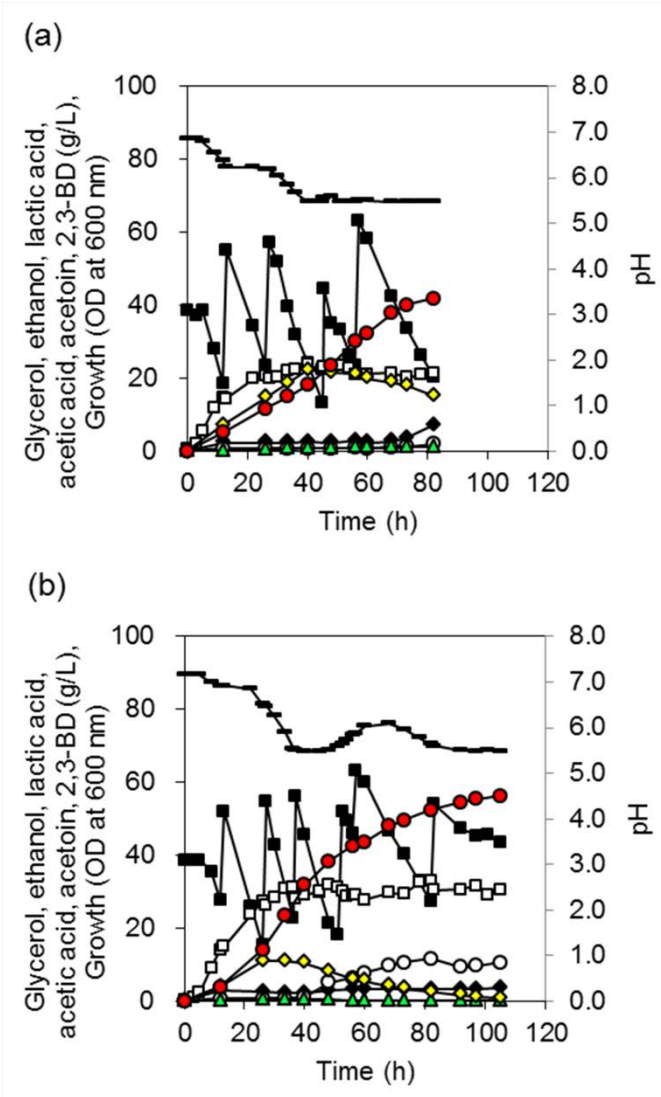

Figure 11. Fed-batch fermentation of glycerol by (a) *R. ornithinolytica* B6 *⊿ldh* and (b) *R. ornithinolytica* B6 *⊿ldh* (pUC18CM-*budABC*) in complex medium under optimized condition; 25 ˚C, 1vvm airflow, 400 rpm, pH control to not below pH5.5; ■, glycerol; □, growth; –, pH; ◊, ethanol; ○, acetoin; ●, 2,3-BD; ♦, acetic acid; △, succinic acid; ▲, lactic acid.

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

Alternatively, a *budABC* overexpression mutant was constructed to increase the carbon flux towards 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) catalyzes the synthesis of α-acetolactate from two moles of pyruvate; α-acetolactate decarboxylase (BudA) converts α-acetolactate to acetoin; and acetoin is converted to 2,3-BD by acetoin reductase (BudC). The overexpression of these genes (*budABC*) was confirmed by SDS-PAGE (Figure 12). There was no difference in growth and products 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, and the 2,3-BD production was enhanced up to 89.5 g/L with the yield of 0.41 g/g and the productivity of 0.75 g/L/h (Table 10). The 2,3-BD production performance was higher than the results obtained with the wild type strain. As expected, the production of acetic acid and ethanol was decreased (Figure 13).

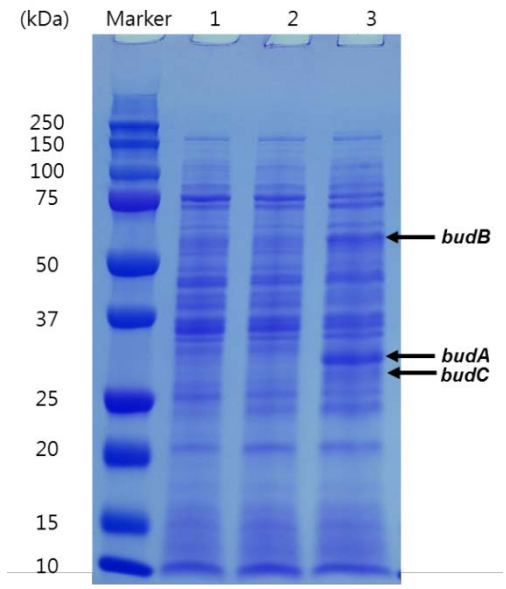

Figure 12. SDS-PAGE analysis of gene overexpression in the *R.ornithinolytica* B6 (pUC18CM-*budABC*). BudA (29 kDa), BudB (61 kDa) and BudC (26 kDa) bands are indicated with arrows; lane 1, *R.ornithinolytica* B6; lane 2, *R.ornithinolytica* B6 (pUC18CM); lane 3, *R.ornithinolytica* B6 (pUC18CM-*budABC*).

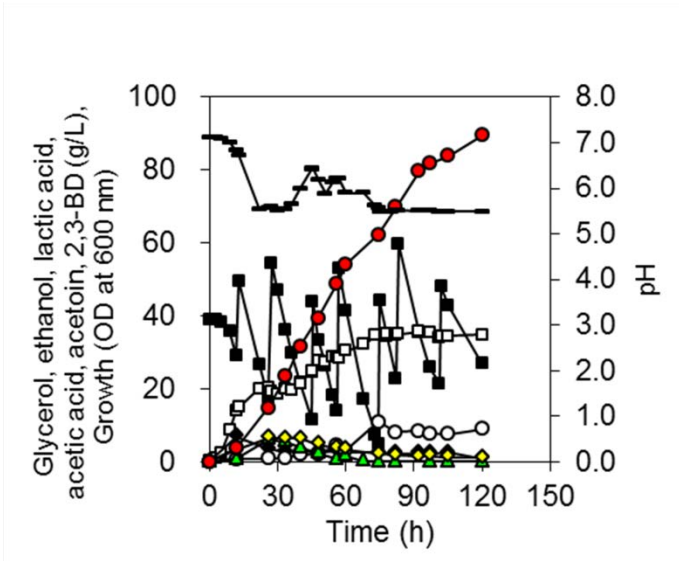

Figure 13. Fed-batch fermentation of glycerol by R. ornithinolytica B6 (pUC18CM-budABC) in the complex medium at 25 ˚C and 400 rpm with the lower pH limit of 5.5; ■, glycerol; □, growth; –, pH; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid.

4.4.7. 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 non-glycerol [MONG], inorganic salts, and ash) (Luo et al., 2016) and these impurities have an inhibitory effect on cell growth, and production of metabolites (Ito et al., 2005; Samul et al., 2014).

To investigate the feasibility of 2,3-BD production from crude glycerol, crude glycerol (AK) and the corresponding pretreated crude glycerol (AK-T) were tested. The compositions of the AK and AK-T are shown in Table 6. Pretreatment was done according to the method of Moon et al. (2010) by which soap and free fatty acids are removed. Although it was lower than when pure glycerol was supplied (16.7 g/L of 2,3-BD), the comparable titer of 2,3-BD was produced as a main product with AK (13.5 g/L) which is higher titer than that with AK-T (12.3 g/L) as shown in Figure 14.

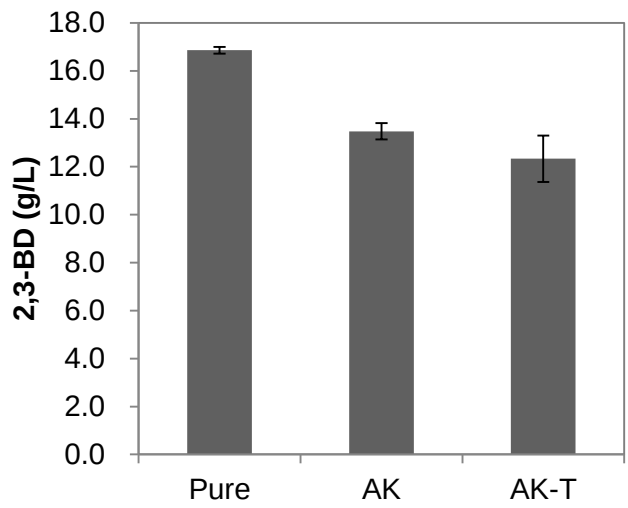

Figure 14. 2,3-BD production of *R. ornithinolytica* B6 using crude glycerol (AK) and the corresponding pretreated crude glycerol (AK-T)

Based on the flask test, AK was selected as feed stock. When AK was supplied as a 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), 65.4 g/L 2,3-BD were produced after 168.5 g/L glycerol consumption during 86.5 hours at the yield of 0.39 g/g and the productivity of 0.76 g/L/h (Table 10 and Figure 15a). The produced 2,3-BD titer was approximately 73.0% of that obtained with pure glycerol using *R. ornithinolytica* B6 (pUC18CM-*budABC*) (Table 10). This low 2,3-BD production with crude glycerol could be the result of the accumulation of inhibitory impurities (present in the crude glycerol) due to the repeated feeding of crude glycerol. Another possible reason could be the marginal pH fluctuations shown in Figure 15. The pH of 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 contrast to the flask test, the 2,3-BD concentration and yield were enhanced up to 78.1 g/L and 0.42 g/g with supply AK-T, respectively, compared to the case of using crude glycerol AK (Figure 15b). Production of 2,3-BD was prolonged up to 126 hours probably due to the removal of impurities through the pretreatment. Furthermore, pH fluctuation and lactic acid formation/consumption were clearly observed (Figure 16).

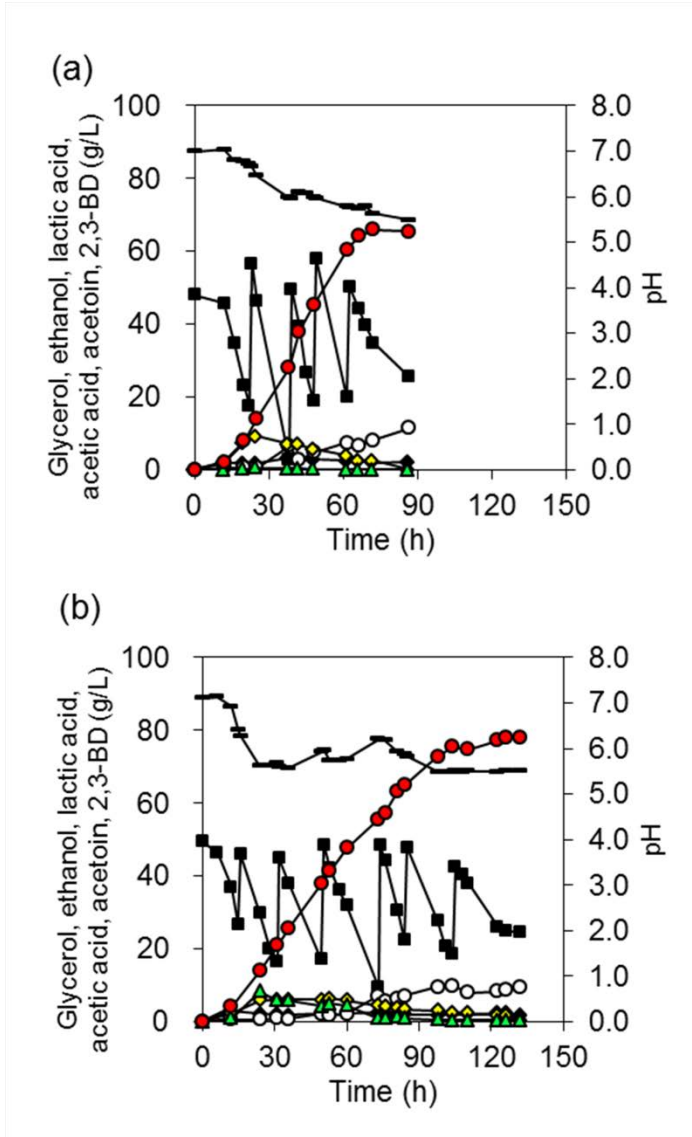

Figure 15. Fed-batch fermentation using (a) crude glycerol, AK and (b) pretreated crude glycerol AK-T by *R. ornithinolytica* B6 (pUC18CM-*budABC*) in complex medium at 25 ˚C and 400 rpm with the lower pH limit of 5.5; ■, glycerol; –, pH; ●, 2,3-BD; ♦, ethanol; ○, acetoin; ♦, acetic acid; ▲, lactic acid.

Table 11. 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[1]						
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[*]	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[2]						
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	79.3	0.40	0.64	This study
R. ornithinolytica B6 (pUC18CM-*budABC*)	Pure glycerol	Fed-batch	89.5	0.41	0.75	This study
	Crude glycerol	Fed-batch	65.4	0.39	0.76	This study
	Pretreated crude glycerol	Fed-batch	78.2	0.42	0.59	This study

[1] possessing 1,3-PD synthesis pathway, [2] deficient in 1,3-PD synthesis ability, [*] *pduC* and *ldhA*-deleted mutant of *K. oxytoca* M1

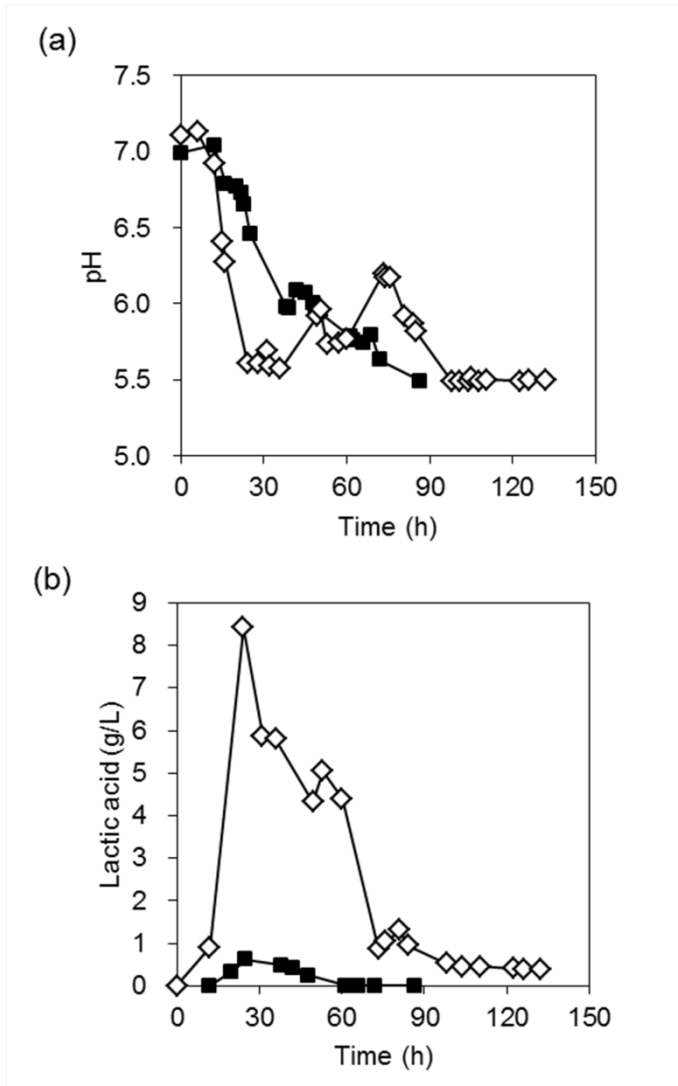

Figure 16. Comparison of (a) pH and (b) lactic acid production between AK (■) and AK-T (◊). Fed-batch fermentation was carried with *R. ornithinolytica* B6 (pUC18CM-*budABC*) at 25 ˚C and 400 rpm with the lower pH limit of 5.5 in the complex medium.

4.5. Discussion

R. ornithinolytica B6 has physiological characteristics distinguished from other 2,3-BD producers. First, there is no glycerol dehydratase in the genome of *R. ornithinolytica* B6. This might support the lack of 1,3-PD synthesis 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 & 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 finding the optimum temperature in a flask test, the aeration condition and pH control mode were investigated in fed-batch fermentation for improving 2,3-BD production of *R. ornithinolytica* B6. Oxygen supply and pH were important factors for 2,3-BD production. Generally, a suitable dissolved oxygen level and pH for 2,3-BD production are depending on the strains and substrates used (Celinska & Grajek, 2009). Therefore, the optimal oxygen supply level was tested under different agitation speeds (200, 300, 400, and 500 rpm). The cell growth and 2,3-BD production was increased with increasing agitation speed up to 400 rpm. However, the fermentation was terminated quickly at 500 rpm. It could be caused by oxidative stress due to excessive oxygen supply. During microbial respiration, molecular oxygen could be converted to reactive oxygen

species, which causes damage to cell (Baez & Shiloach, 2013). After determine agitation speed as 400 rpm, the pH control strategy was set by testing three different pH control conditions; i) no pH control, ii) pH control with the lower limit of 5.5, iii) pH control with the lower limit of 6.0. Because the 2,3-BD production was quickly ended likely due to a significant pH drop (Fig 8c), pH control was necessary for preventing acidic condition. The reason why pH was not strictly controlled at a certain level is to permit pH fluctuation based on the previous finding that the pH fluctuation has a positive effect on 2,3-BD production (Petrov & Petrova, 2009). 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 N NaOH to raise pH with ΔpH of 1 every 12 hours, and the final 2,3-BD concentration during 150 hours of fermentation was 70.0 g/L, which was 1.33-fold higher than that of no pH control case (52.5 g/L). Therefore different lower limit was set for investigating pH profile and its effect on 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 1.7-fold with the pH control above 5.5 and 6.0. Meanwhile, 2,3-BD yield and productivity were higher at the lower pH limit of 5.5 than at the lower pH limit of 6.0, probably due to the range of pH fluctuations. The ΔpH was larger at pH control above 5.5 (ΔpH = 0.5~0.8) than at pH control above 6.0 (ΔpH = 0.2~0.3). Moreover, it was found that the pH fluctuation was related to the production of lactic acid through the *ldh* deletion experiment. This phenomenon caused by lactic

acid/2,3-BD alteration may be due to expression level of lactate dehydrogenase and α-acetolactate or enzyme activities depending on pH. Lactic acid and α-acetolactate are synthesized from pyruvate by lactate dehydrogenase and α-acetolactate synthase, respectively. Park et al. (2013a) reported the metabolic flux distribution of *K. oxytoca* at pH 5.5 and pH 7.0. According to the metabolite profile and metabolic flux analysis data, the lactic acid flux decreased from pH 7.0 to 5.5 but fluxes toward 2,3-BD increased. Consequently, the pH fluctuation observed in this study explained by the previous study about pH affecting metabolic flux redistribution and the fact that when pH is above 5.5, pH is decreased by lactic acid production and when pH is reached 5.5, pH is increased by 2,3-BD production and lactic acid reassimilation. B6 strain has no pathway for 1,3-PD, thus deletion of NADH consuming by-products has negative effect. However, by applying the lower pH limit strategy to allow pH fluctuations, intracellular redox balance could be regulated by lactic acid/2,3-BD alterations. Consequently, fermentation was prolonged and 2,3-BD production was increased.

Table 11 shows 2,3-BD production using glycerol (pure or crude) as a 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 concentration was relatively low (27.3 ~ 33.6 g/L), and further investigations such as pH control strategies and mutant construction were not reported. In this study, high production of 2,3-BD without 1,3-PD synthesis 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 11).

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.

Conclusions

2,3-Butanediol (2,3-BD) is a very useful chemical with wide ranges of industrial applications. There are many studies to produce 2,3-BD using wild type and metabolically engineered microorganisms. This study aims to expand the spectrum of 2,3-BD producing bacteria by isolation of a non-pathogenic 2,3-BD producer.

In Chapter 2, identification and confirmation of a 2,3-BD producer by using various carbon sources were conducted. The isolate from a soil sample of Baegun Mountain in Korea was designated as *Raoultella ornithinolytica* B6, which is classified as biosafety level 1. Moreover, in an optimum temperature test, *R. ornithinolytica* B6 did not grow at normal body temperature (37 °C). And the low the temperature (25 °C), *R. ornithinolytica* B6 grows well. Although there is no direct evidence from the pathogenicity test, it is difficult to see that B6 has pathogenicity, given the fact that it does not grow well at 37 °C. *R. ornithinolytica* B6 produces 2,3-BD as a main product by using hexoses (glucose, galactose, fructose), xylose, sucrose and glycerol as a sole carbon source. The characteristics of producing 2,3-BD by using various carbon sources implies possibility of *R. ornithinolytica* B6 to be used for lignocellulosic biomass fermentation in further study. Moreover, the B6 strain could produce 2,3-BD using glycerol, which is a promising renewable resource. Consequently, the newly isolated bacteria, *R. ornithinolytica* B6 can be considered as a potential candidate for 2,3-BD production.

In Chapter 3, based on Chapter 2, high production of 2,3-BD with *R. ornithinolytica* B6 was achieved by optimizing fermentation conditions and metabolic engineering. For improving 2,3-BD production, optimization of a fed-

batch fermentation conditions was performed. Through adapting the “two-stage pH control” strategy, an improvement of 2,3-BD production by a 1.5-fold compared with no-pH control was achieved. The pH control strategy maintains the pH at 5.5 after the pH naturally decreased from initial pH 7. Aeration was controlled by agitation with 1vvm airflow. The highest concentration of 2,3-BD (68.3 g/L) was produced at 400 rpm. Further improvements of 2,3-BD production in titer (112.2 g/L), yield (0.38 g/g) and productivity (1.35 g/L/h) were achieved by overexpressing the homologous *budABC* genes, the 2,3-BD producing related genes involved in the conversion of pyruvate to 2,3-BD. Comparing this result with previously reported 2,3-BD production (Table 1), *R. ornithinolytica* B6 can be considered as a potential candidate for high 2,3-BD production with expectation of enhanced 2,3-BD production.

The efficient 1,3-BD-free 2,3-BD production by using a renewable resource, crude glycerol was conducted in Chapter 4. *R. ornithinolytica* B6 has no 1,3-PD synthesis pathway; therefore, the cost for separation/purification of 2,3-BD in industrial processes can be reduced. Optimum fermentation conditions for 2,3-BD production from glycerol were found to be 400 rpm and pH control with lower limit of 5.5. Notably, significant pH fluctuations caused by lactate/2,3-BD alteration with simply controlling lower pH limit at 5.5 has a positive effect on 2,3-BD production. In fed-batch fermentation under the optimized conditions, *R. ornithinolytica* B6 (pUC18CM-*budABC*) effectively produced 2,3-BD using pure glycerol and crude glycerol up to 89.5 g/L and 78.1 g/L, respectively, providing a feasible 1,3-PD-free 2,3-BD production process.

This study demonstrates the high 2,3-BD production ability of the newly

isolated bacterium, *R. ornithinolytica* B6 from glucose and crude glycerol, hence extends a microbial spectrum of 2,3-BD producers over previously known 2,3-BD producers.

Based on this study, further studies on i) carbon catabolic repression in *R. ornitinoytica* B6 for utilizing derived from lignocellulosic biomass and marine biomass and ii) evolutionary engineering to increase an optimum temperature for 2,30BD production will promote practical application of *R. ornithinolytica* B6.

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Appendix

Data S1. 16S rRNA gene sequence of *R.ornithinolytica* B6 (Accession number KT445983)

GCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG

CCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAAC

GTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTG

CCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACG

ATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACAC

GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC

GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTG

TAAAGTACTTTCAGCGAGGAGGAAGGCATTAAGGTTAATAACCTTAGTG

ATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC

GCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG

CGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACC

TGGGAACTGCATTTGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTA

GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGG

TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC

GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGA

TGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGC

GTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG

AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG

CAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAG

ATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCG

TCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC

CTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCC

AGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCT

TACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCG

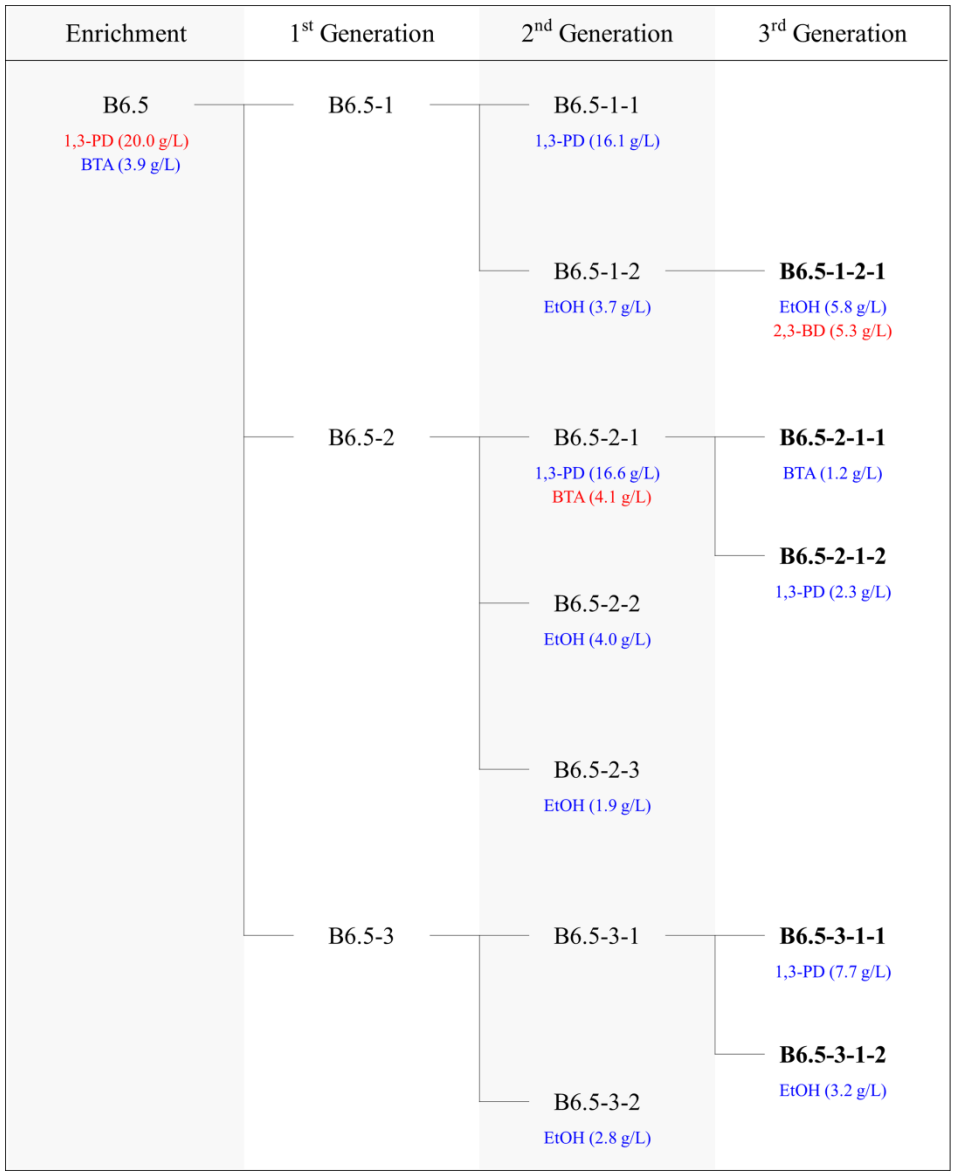
ACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTG

GAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATC

AGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA

CACCATGGGAGTGGGTTGCAAAAGAAGTA

Figure S1. Isolation procedure from soil sample under anaerobic condition, the blue and red color indicate main product when supplied glycerol and glucose as a sole carbon source, respectively. Single colony samples are shown in bold; EtOH, ethanol; BTA, butyric acid; 2,3-BD, 2,3-butanediol; 1,3-PD, 1,3-propanediol.



국 문 초 록

석유 고갈과 이로 인한 석유 산업을 기반으로 생산하는 화학물의 경제성, 그리고 환경 문제에 대한 관심이 증가함에 따라 생물학적 방법을 통한 유용물질 생산에 대한 관심은 날로 증가하고 있다.

그 중에서도 2,3-butnaediol (2,3-BD)은 생물공학적 방법을 이용해 생산이 가능하며 그 활용도가 높아 생산 가능한 미생물과 발효 공정에 대한 연구가 활발히 진행되어 왔다.

그러나 독보적으로 높은 생산성을 보이는 미생물은 *Klebsiella* 종에 속하는 박테리아로 해당 미생물들은 병원성을 갖고 있어 산업적 측면에서 안전성 문제를 갖고 있다.

한편, 바이오디젤 생산 증가에 따라 부산물로 생산되는 글리세롤의 가격이 꾸준히 낮아지고 있음에도 불구하고, 고수율의 2,3-BD 생산을 위한 탄소원으로 글리세롤을 채택하여 진행한 연구는 당류를 활용한 연구에 비해 상대적으로 미진하다.

이에 따라 본 연구는 당 뿐만 아니라 글리세롤을 탄소원으로 이용하여 효율적인 2,3-BD의 생산이 가능한 새로운 미생물을 제시함으로써 2,3-BD의 산업적 생산에 적용 가능한 탄소원의 다양화 및 균주의 다양화를 목적으로 한다.

이를 위해 유류 오염 토양 샘플에서 미생물을 분리, 동정하였으며, *Raoultella ornithinolytica* B6 라 명명하였다. 본 균주는 다양한 6탄당

(포도당, 과당, 갈락토스)과 5탄당인 자일로스, 이당류인 수크로스, 그리고 글리세롤을 활용하여 2,3-BD를 주요 생산물로 생산할 수 있다는 것을 확인 하였다.

이에 *R. ornithinolytica* B6 균주를 이용한 2,3-BD 생산성 향상을 위해 먼저 최적 발효조건을 탐색하였다. 당류 중에서는 가장 높은 수준으로 2,3-BD를 생산할 수 있도록 하는 기질인 포도당을 주요 기질로 하여 최적 배양 온도를 확인 하고, 유가식 회분배양 조건을 확립하였다.

먼저 pH 조건을 확립하였는데, 초기 pH 7.0 에서 배양하여 5.5에서 유지하는 이단계 조절 전략을 사용하여 2,3-BD 생산성을 향상시켰다. 그 다음으로는 교반속도를 조절하여 공기공급량을 조절하였다. 결과적으로 400 rpm의 속도로 교반할 때 2,3-BD (68.3 g/L)를 가장 높게 생산하는 것을 확인할 수 있었다.

다음 단계로 2,3-BD 생산량을 높이기 위해 대사공학적 방법을 통한 균주 개발을 수행하였다. 피루빈산의 2,3-BD로의 전환에 관여하는 세 개 유전자 (*budA*, *budB*, *budC*)가 과발현 된 균주 *R. ornithinolytica* B6(pBbA5c-*budABC*)는 앞서 확립한 최적 발효조건 하에 최종적으로 112.5 g/L의 2,3-BD를 생산하여 기존에 알려진 2,3-BD 생산 균주와 견주어 손색이 없음을 증명하였다.

한편, *R. ornithinolytica* B6 의 유전체 분석을 통해 글리세롤을 기질로 공급 시, 2,3-BD 분리 정제에 문제가 되는 부산물인 1,3-propanediol (1,3-PD)을 생산하는 대사경로를 갖고 있지 않은 것

이 확인되었다. 이에 따라 높은 경제성을 가진 재생 가능한 자원인 폐 글리세롤을 기질로 공급하여 2,3-BD를 생산하고자 하는 연구를 진행하였다.

전체적인 연구의 흐름은 당을 공급했을 때와 같이 최적 배양 온도를 확인하고 유가식 회분배양 조건을 pH 조절과 공기 공급 측면에서 확립하였는데, 당을 공급했을 때와 다른 점은 pH 조절 전략으로 pH 5.5 에서 고정하는 것이 아니고 pH 5.5를 하한값으로 설정하여 5.5 이상에서의 pH 변동을 허용하였다. 이는 인위적인 pH 의 변화가 2,3-BD의 생산성 향상에 영향을 미친다는 기존의 연구를 바탕으로 하지만, 해당 연구에서는 인위적으로 일정 시간 간격을 두고 일정한 폭으로 pH를 올려서 pH에 맥을 유발하였다면, 본 연구에서는 하한선의 설정만으로 자동적으로 pH의 파동이 유발되었다는 점에서 기존 연구와 차별화 된다. 그리고 이러한 pH 파동이 1차 대사산물로 생산되는 젖산에 의해 유발 됨을 lactate dehydrogenase 유전자의 삭제를 통해 규명하였다.

이 과정을 통해 확립한 발효 조건에서, *budABC* 과발현 균주 *R. ornithinolytica* B6(pUC18CM-*budABC*)를 이용한 글리세롤 발효가 수행되었다. 순수 글리세롤을 기질로 하여 89.5 g/L 의 2,3-BD를 생산하였으며, 폐 글리세롤을 기질로 하여 78.1 g/L를 생산하였다. 상술한 바와 같이 본 균주는 1,3-PD를 생산하지 않으므로 글리세롤을 탄소원으로 하여 2,3-BD를 생산하는 경우, 기질의 경제성뿐 아니라 분리정제 공정에 있어서의 경제성까지 확보할 수 있다.

본 연구는 기존에 발표되었던 균주와 다르게 생물안전도 1의 미생물인 *Raoultella* 에 속하는 균주를 2,3-BD의 산업적 생산을 위한 후보 균주로 제시함으로써, 2,3-BD 생산 균주 범위를 확장시켰으며, 최초로 *R. ornithinolytica* 의 대사공학 및 발효 조건을 확립하였음에 그 의의가 있다.

주요어 : 2,3-BD, *Raoultella ornithinolytica, budABC,* 유가식 회분 배양 조건 최적화, 대사공학, 폐 글리세롤

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