A Thesis for the Degree of Master of Science

Molecular cloning and expression of α-acetolactate synthase and α- acetolactate decarboxylase for 2,3-butanediol production in Pdc-deficient *Saccharomyces cerevisiae*

Pdc 결여 효모로부터 2,3-부탄다이올 생산 증진을 위한 α-acetolactate synthase 와 α- acetolactate decarboxylase 의 최적 발현 시스템 구축

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Advisor : Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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指導敎授 徐 鎭 浩

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農生命工學部 食品生命工學 專攻
崔 明 賢

崔 明 賢 의 農學碩士 學位論文을 認准함
2015年 8月

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ABSTRACT

2,3-Butanediol (2,3-BD) is a chemical compound with extensive industrial applications. Especially, 2,3-BD could be converted to valuable chemicals by dehydrogenation, esterification, ketalization and dehydration. It has been used as drugs, solvents, flavoring agents, cosmetic and food products. Although biotechnological production of 2,3-BD mainly uses bacteria, most bacteria used for 2,3-BD production are classified as potentially pathogenic microbes, which makes difficult industrial-scale production of 2,3-BD in terms of safety regulations. As an alternative, 2,3-BD production by a GRAS (Generally Regarded As Safe) microorganism *Saccharomyces cerevisiae* would be suitable.

As *S. cerevisiae* naturally produces ethanol, a pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* (SOS4) was constructed to eliminate ethanol production. The 2,3-BD biosynthetic pathway was intensified for redirecting pyruvate toward 2,3-BD production. Since

the (Pdc)-deficient *S. cerevisiae* has a low 2,3-BD yield and productivity, it is necessary to improve both 2,3-BD yield and productivity by optimizing the biosynthetic pathway from pyruvate to 2,3-BD.

First, bacteria which produce a high 2,3-BD yield were investigated. *Klebsiella pneumoniae, K. oxytoca*, and *Enterobacter aegrogenes* which can produce 2,3-BD at high yield and productivity were selected. The genes coding for acetolactate synthase (ALS) and acetolactate decarboxylase (ALDC) from *K. pneumoniae, K. oxytoca*, and *E. aegrogenes* were cloned and introduced to the SOS4 strain which is also able to overexpress the *BDH1* gene. The yeast strain containing both the ALS gene and ALDC gene from *B. subtilis* and the innate *BDH1* gene (BD_BS) was used as the control strain. A batch fermentations with 100 g/L glucose was carried out under oxygen-limited conditions. The 2,3-BD yield and productivity of the control

strain were still higher than other strains.

Second, the SOS4 strains containing the ALS gene from *B. subtilis* and the ALDC gene from *K. pnuemoniae, K. oxytoca*, and *E. aerogenes* and overexpression of the innate *BDH1* gene were constructed and tested for performance of 2,3-BD production in batch fermentations with 100 g/L glucose under oxygen-limited conditions. The 2,3-BD yield and productivity of the BD_BS_EA strain containing ALS from *B. subtilis* and ALDC from *E. aerogenes* and overexpressing the innate *BDH1* gene were slightly higher than the control strain. To explore the potential ability of 2,3-butanediol production between the control and the BD_BS_EA strains as a 2,3-butanediol producer, fed-batch fermentations was carried out through intermittent addition of glucose under the optimum aeration condition. The final concentration of 2,3-butanediol of the BD_BS_EA strain was 132.4 g/L with a 2,3-butanediol yield (0.34 g 2,3-butanediol/g glucose) and volumetric

productivity (0.41 g 2,3-butanediol/L·h), corresponding to 28% increase in yield and 24% increase in productivity.

Finally, in order to analyze why the BD_BS_EA strain is better than the control strain, the specific activity of ALDC from *E. aerogenes* was compared with that ALDC from *B. subtilis*. The ALDC was purified by affinity chromatography using the histidine tag. Specific activity of ALDC from *E. aerogenes* was found to be 250.4 mU/mg protein, which is higher by 2.5-folds than ALDC from *B. subtilis*. Although ALDC from *E. aerogenes* possessed a low substrate affinity (*Km*), Vmax and *kcat*/*Km* of ALDC were higher by 5-folds, 1.5-folds than ALDC from *B. subtilis* respectively. These results suggested that the BD_BS_EA strain is suitable for producing 2,3-BD for industrial applications.

Keywords : 2,3-butanediol, pyruvate decarboxylase (Pdc)-deficient *S. cerivisiae*, fed-batch, α-acetolactate decarboxylase

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I. INTRODUCTION

1. 2,3-Butanediol

2,3-Butanediol is a four-carbon compound with molecular weight of 90.12 kDa. The chemical formula for 2,3-butanediol is $CH_3CH(OH)CH(OH)CH_3$ and has three isomeric forms: *D*-(-)-, *L*-(+)- and *meso*-. (Fig. 1). Other name is 2,3-buylene glycol, dimethylethylene glycol or 2,3-dihydroxybutane (Syu, 2001; Celinska and Grajec, 2009).

Because of low freezing point of -60℃, 2,3-buatnediol is used as anti-freeze agents. 2,3-Butanediol can be converted to other beneficial chemicals by dehydrogenation, esterification, ketalization and dehydration. So 2,3-butanediol is used in various industrial product: Food additives, moisturizers, drugs, cosmetic products and lotions, etc. In addition, 2,3-butanediol is also utilized as a flavoring agent in food products when converted to a diacetyl by dehydrogenation(Garg and Jain, 1995). In addition, 2,3-butanediol can be converted to 1,3-butadien that is material used in the production of synthetic rubber (Syu, 2001).

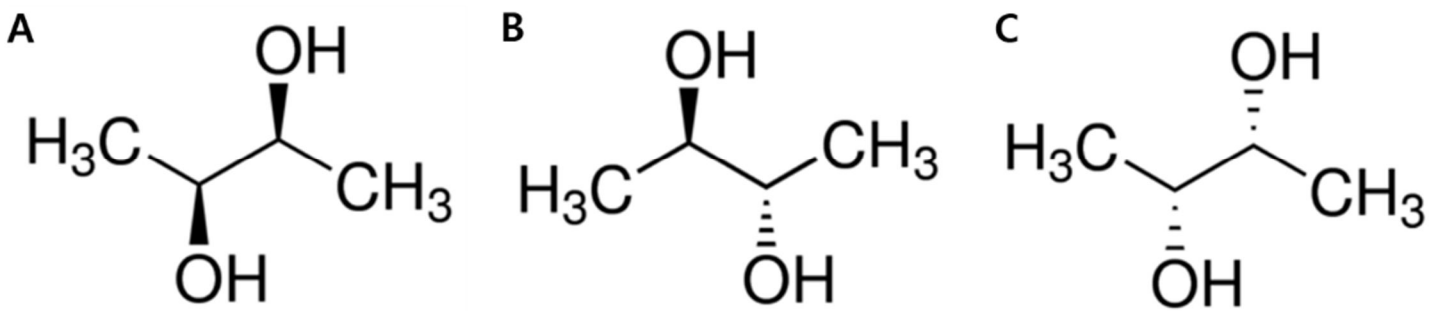

Figure 1. The stereoisomers of 2,3-butanediol.

(A) *L*-(+)-2,3-butanediol, (B) *meso*-2,3-butanediol, (C) *D*-(-)-2,3-butanediol.

2. Metabolic pathway

The biosynthesis of 2,3-butanediol from glucose is produced some intermediate compounds such as α-acetolactate, acetoin and diacetyl in a fermentation process. First, glucose is converted into pyruvate by glycolysis. After decarboxylation, pyruvate can be converted into α-acetolactate by α-acetolactate synthase (ALS). α-Acetolactate can be converted into acetoin by α-acetolactate decarboxylase (ALDC) under anaerobic conditions. If oxygen is enough, α-acetolactate can be converted into diacetyl by spotntaneous decarboxylation. Then, acetoin dehydrogenase can convert diacetyl to acetoin. Finally, acetoin can be reduced to 2,3-butanediol by butanediol dehydrogenase (BDH) (Fig. 2) (Celinska and Grajek, 2009). All enzymes and intermediates included in the 2,3-butanediol pathway are produced during the log and stationary phases of fermentation, when oxygen-limiting conditions exist (Mallonee and Speckman, 1988)

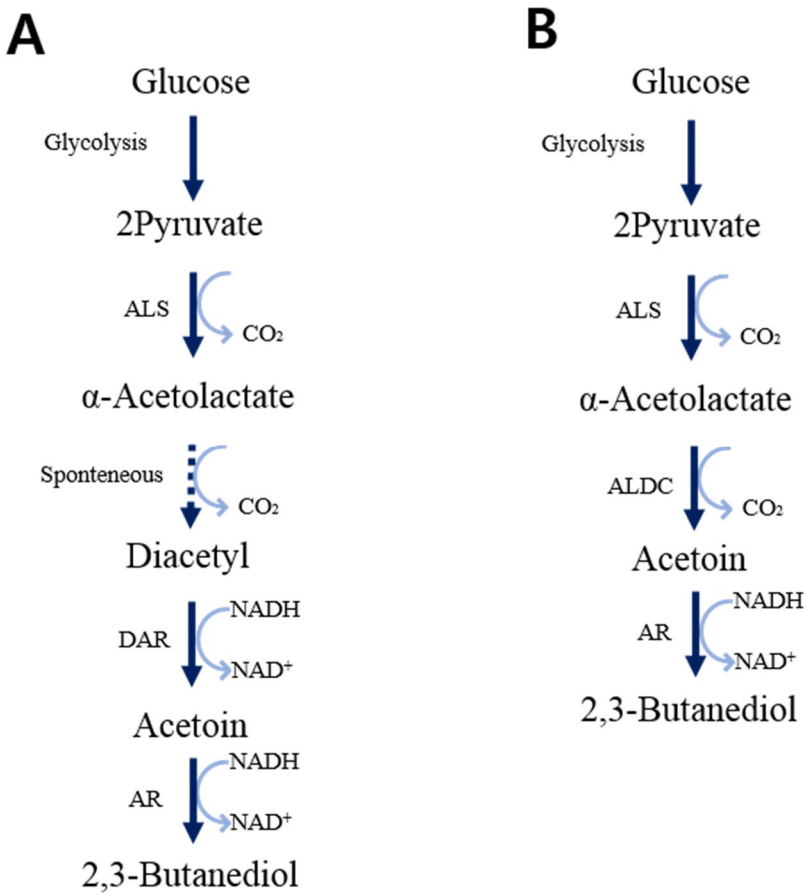

Figure 2. Metabolic pathways of 2,3-butanediol from glucose. Two alternative pathways of acetoin synthesis (A and B) are pictured. ALS : α-acetolactate synthase; ALDC : α-acetolactate decarboxylase; DAR : diacetyl reductase; AR : acetoin reductase.

3. 2,3-Butanediol production in bacteria

A number of bacteria species can produce 2,3-butanediol but only a few do so in what might be considered significant quantities (Ji et al. 2011). Until now, *B. polymyxa, K. pnuemoniae, K. oxytoca, S. marcescens* and *E. aerogenes* efficiently produce 2,3-butanediol with high yield and productivity (Table 1) (Celinska and Grajek, 2009).

Bacteria be able to produce 2,3-butanediol via a mixed acid fermentation along with by-products such as acetate, lactate, formate, succinate and ethanol (Fig. 3) (Magee and Kosaric, 1987; Maddox, 1996). Four key enzymes are included in 2,3-butanediol production from pyruvate: α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), diacetyl reductase (DAR) and butanediol dehydrogenase (BDH) (Celinska and Grajek, 2009).

Most bacteria related to 2,3-butanediol production is classified as pathogenic microbes (Class Ⅱ). Therefore the use of safe microorganisms is required in order to substitute for these pathogenic 2,3-butanediol producing bacteria (Kim and Timmusk, 2013; Li et al., 2013)

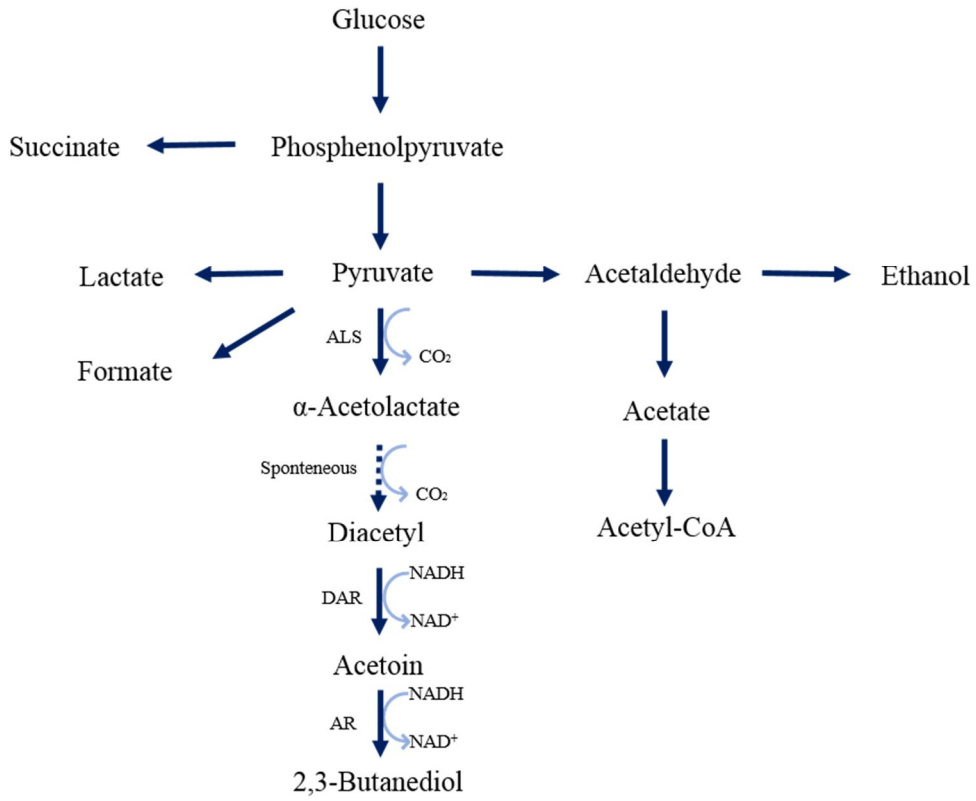

Figure 3. Mixed acid 2,3-butanediol pathway in bacteria. ALS : α-acetolactate synthase; DAR : diacetyl reductase; AR : acetoin reductase.

Table 1. Microbial production of 2,3-butanediol (Celinska and Grajek, 2009)

Strains	Substrates	Methods	2,3-BD concentration (g/L)	Yield (g/g)	Productivity [g/(L·h)]	References
Paenibacillus polymyxa	Sucrose	Fed-batch	111.0	-	2.05	Hassler et al., 2012
Klebsiella pneumoniae	Glucose	Fed-batch	150.0	0.43	4.21	Ma et al., 2009
Klebsiella oxytoca	Glucose	Batch	95.5	0.49	1.74	Ji et al., 2009
Enterobacter aerogenes	Glucose	Fed-batch	110.0	0.49	5.40	Zeng et al., 1991
Serratia marcescens	Sucrose	Fed-batch	152.0	0.41	2.67	Zhang et al., 2010
Bacillus subtilis	Glucose	Batch	2.5	0.31	0.27	Moes et al., 1985
Saccharomyces cerevisiae	Glucose	Batch	2.3	0.03	0.11	Ng et al., 2012

4. 2,3-Butanediol production in *S. cerevisiae*

Several yeasts able to synthesize 2,3-butanediol and *S. cerevisiae* is known to produce a trace amount of 2,3-butanediol naturally, but the 2,3-butanediol yield and productivity are extremely poor (Garg and jain, 1995; Celinska and Grajek, 2009).

In *S. cerevisiae*, there are two pathways to produce 2,3-butanediol (Fig. 4). One pathway is that pyruvate is converted into α-acetolactate by α-acetolactate synthase (*ILV2*) included in the valine and isoleucine synthetic pathway. Because α-acetolactate decarboxylase does not exist in *S. cerevisiae* unlike bacteria, α-acetolactate cannot be enzymatically decarboxylated into acetoin. Therefore α-acetolactate can be converted into diacetyl by spontaneous decarboxylation in the existence of oxygen. Diacetyl is reduced into acetoin by diacetyl reductase. Finally, acetoin is converted into 2,3-butanediol by butanediol dehydrogenase (*BDH1*). The other pathway is pyruvate decarboxylase is known to condense pyruvate or acetaldehyde into acetoin. (Ehsani et al., 2009; Guymon and Crowell, 1967).

2,3-Butanediol production by wild type *S. cerevisiae* has not been done extensively. Previous attempts have concentrated on improving a flavor in beer and wine fermentations rather than producing 2,3-

butanediol as the main product. Recently, research concentrated on production of 2,3-butanediol by engineered *S. cerevisiae* was reported. A strategy for gene deletion was performed by using *in silico* genome scale metabolic analysis. Deletion of *ADH1*, *ADH3* and *ADH5* genes coding for alcohol dehydrogenase resulted in an improvement of 2,3-butanediol production by a 55 fold with reduced ethanol production compared to the wild type strain. The highest 2,3-butanediol titer and yield are 2.29 g/L, 0.113 g 2,3-butanediol/g glucose) respectively. Its was obtained by the engineered *S. cerevisiae* deficient in the ADH genes in anaerobic conditions (Ng et al., 2012)

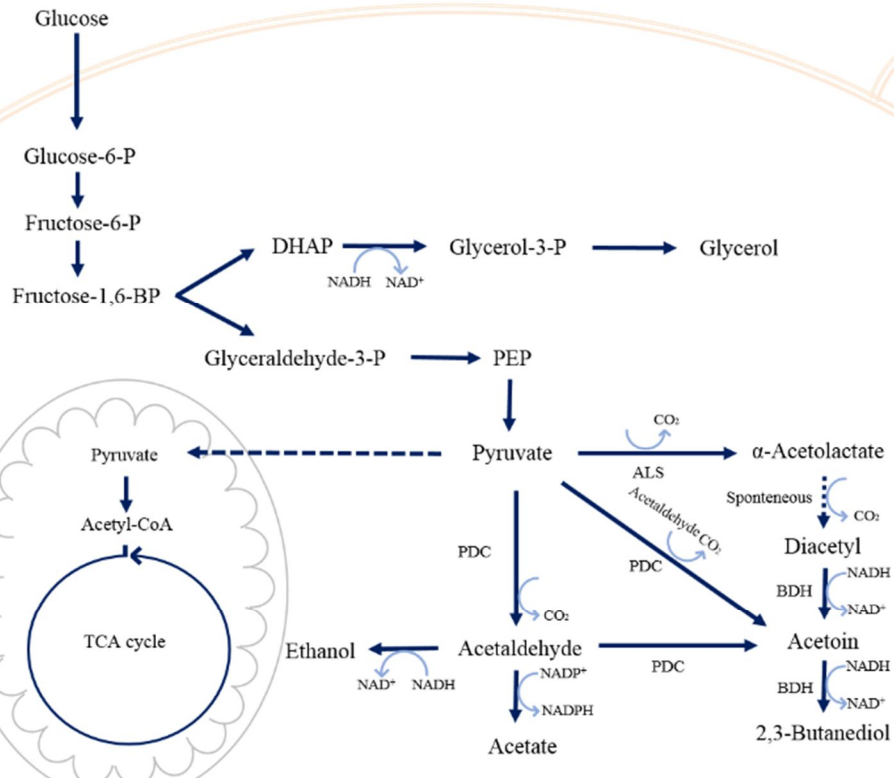

Figure 4. 2,3-butanediol biosynthesis in *S. cereivisae*. ALS : α-acetolactate synthase; PDC : pyruvate decarboxylase; BDH : 2,3-butanediol dehydrogenase

5. 2,3-Butanediol production in pyruvate decarboxylase-deficient *S. cerevisiae*

In *S. cerevisiae*, which is traditionally used to ferment sugar to ethanol, is suitable as a host for the production of therapeutic proteins or chemicals by metabolic engineering. Because the yield of the desired product should be maximized in respect of the economy, it is essential for redirect carbon fluxes away from ethanol production towards the desired product. In order to minimize ethanol production and to maximize the production of desired products, pyruvate decarboxylase (Pdc)-deficinet *S. cerevisiae* has been utilized as the production strain for lactic acid, glycerol and malic acid (Geertman et al., 2006; Ishida et al., 2006; Zelle et al., 2008)

Pyruvate decarboxylase is located at the branch point between the fermentative and respiratory metabolism, and converts pyruvate to acetaldehyde, which is further reduced into ethanol by alcohol dehydrogenase. In *S. cerevisiae*, there are three structural genes (*PDC1, PDC5*, and *PDC6*) included as active pyruvate decarboxylase isoenzymes. Among these genes, disruption of *PDC1* and *PDC5* or all *PDC* genes led to elimination of pyruvate decarboxylase activity

completely (Flikweert et al., 1996).

The Pdc-deficient strains have potential defects for industrial fermentations. Firstly, the Pdc-deficient mutant requires external supplement of two-carbon compounds such as acetate or ethanol for synthesis of cytosolic acetyl-CoA which is required to synthesize lysin and fatty acids (Flikweert et al., 1996; Pronk et al., 1996). Cytosolic acetyl-CoA is synthesized from acetaldehyde via acetate in the cells. However, because of the elimination of Pdc activity, synthesis of acetaldehyde is blocked which leads to the shortage of cytosolic acetyl-CoA. Secondly, the Pdc-deficient mutants showed lower growth rate (h) a glucose-containing medium than the wild type of *S. cerevisiae*. While respiration is necessary for re-oxidation of cytosolic NADH in the Pdc-deficient strains in the absence of ethanol fermentation. Because glucose represses respiration, the Pdc-deficient strains suffer from redox imbalance.

The *PDC1* and *PDC5* genes were deleted in the *S. cerevisiae* D452-2 strain (SOS2). And a two carbon compound-independent and glucose tolerant Pdc-deficient strain (SOS4) was constructed by evolutionary engineering of the SOS2 strain. Genome sequencing of the SOS4 strain revealed a point mutation (A81P) in the *MTH1* gene, leading to an

amino acid convert from alanine to proline (Ala81Pro). The SOS4 strain was reduced to glucose uptake which alleviated the pyruvate accumulation and redox imbalance from eliminating PDC genes activity in the evolved Pdc-deficient strain.

In order to produce 2,3-butanediol in the engineered *S. cerevisiae* (SOS4) strain, *alsS* and *alsD* genes coding for α-acetolactate synthase and α-acetolactate decarboxylase from *B.subtilis* were introduced. Additionally, the endogenous *BDH1* gene coding for 2,3-butanediol dehydrogenase was overexpressed in the SOS4 strain. Therefore the resulting strain (BD4) produced 2,3-butanediol from glucose without ethanol production. The BD4 strain produced 96.2 g/L of 2,3-butanediol from glucose with a yield (0.28 g 2,3-butanediol/g glucose) and a productivity (0.39 g 2.3-butanediol/L·h) in a fed-batch fermentation under optimum conditions (Kim et al., 2013).

In addition to glucose, 2,3-butanediol from cellulosic sugars, such as xylose and cellobiose by engineered yeast have been reported. The *XYL1*, *XYL2*, and *XYL3* genes coding for xylose assimilating enzymes derived from *Scheffersomyces stipites* were introduced into the SOS4 strain to enable xylose utilization. Additionally, the *alsS* and *alsD* gnens from *B. subtilis* and endogenous *BDH1* gene were overexpressed to

increase 2,3-butanediol production from xylsoe. The resulting strain (BD4X) produced 20.7 g/L of 2,3-butanediol from xylose with a yield (0.27 g 2,3-butanediol/g xylose) (Kim et al., 2014). Also, in order to utilize cellobiose, celloextrin transpoter (*cdt-1*) and β-glucosidase (*gh1-1*) from *Neurospora crassa* were expressed in the Pdc-deficient strain SOS2. Additionally, the *alsS* and *alsD* gnens from *B. subtilis* were introduced. The resulting strain (SOS2-CB) produced 5.29 g/L of 2,3-butanediol from cellobiose with a yield (0.29 g 2,3-butanediol/g cellobiose) and a productivity (0.22 g 2.3-butanediol/L·h) (Nan et al., 2014). These results suggest the possibility of producing 2,3-butanediol sustainably and safely from cellulosic sugars.

6. α-Acetolactate decarboxylase

α-Acetolactate decarboxylase (EC 4.1.1.5, also called (S)-hydroxy-2-methyl-2 3-oxobutanoate carboxy-lyase) is an enzyme that converts α-acetolactate directly to acetoin. This enzyme was found in many bacteria but not in yeasts (Fujii et al., 1990). α-Acetolactate decarboxylase is known to occur in a variety of bacteria as e.g. strains of *Enterobacter, Aeromonas, Streptococcus, Serratia, Leuconostoc* and *Bacillus*. These organisms depend at least to some extent on such enzymes for the formation of acetoin and 2,3-butanediol. In the cheese industry, the ability of *S. diacetylactis* and strains of *Leuconostoc* to synthesize acetoin, 2,3-butanediol and diacetyl is utilized for flavor development in cheese. However strains of *B. polymyxa, E. aerogenes* and *A. hydrophila* have been utilized for synthesis of 2,3-butanediol on an industrial scale. Therefore α-acetolactate decarboxylase is regarded as a key enzyme in the bio-based pyruvate conversion processes to produce 2,3-butanediol (Godtfredsen and Ottesen, 1982).

7. Research objectives

This study was focused on the production of 2,3-butanediol by metabolically engineered *S. cerevisiae*. The specific objectives of this research are listed:

1) To search for heterologous α-acetolactate synthase and α-acetolactate decarboxylase for 2,3-butanediol production in *S. cerevisiae*.

2) To enhance 2,3-butanediol production by optimizing the 2,3-butanediol biosynthetic pathway in *S. cerevisiae*.

3) To improve yield and productivity of 2,3-butanediol by fed-batch fermentation in *S. cerevisiae*

4) To measure biochemical properties of α-acetolactate decarboxylase.

Ⅱ. Materials and Methods

1. Reagents

All chemicals used were of reagent grade. Glucose, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), ethyl-2-acetoxy-2-methyl-acetoacetate, α-naphthol, imidazole and 2,3-butanediol were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Bacto-peptone, bacto-trypton, yeast extract and bacto-agar were obtained from Difco (Detroit, MI., USA); 0.5 mm dia. Glass beads from BioSpec (Bartlesville, OK, USA) maleic acid from ACROS organics (Geel, Belgium); creatine from Santa Cruz Biotechnology (Dellas, Texas, USA); ethanol from Merck (Darmstadt, Germany); NaCl, NaOH, sodium phosphate, HCl and H_2SO_4 from Duksan (Ansan, Korea).

2. Strains and plasmids

2.1. Strains

E. coli TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used for the propagation and preparation of plasmid DNA.

S. cerevisiae D452-2 [*Matα, leu2 his3 ura3 can1*] and the pyruvatedecarboxylase (Pdc)-deficient *S. cerevisiae* D452-2 strain (SOS4) was used as host strains for the expression of the genes involved in 2,3-butanediol biosynthetic pathway. (Table 2)

S. cerevisiae D452-2 (Nikawa et al., 1991) was used for protein purification. The SOS4 and BD_BS were constructed by S. J. Kim at Seoul National University in Korea (Kim et al., 2013). Other strains in Table 2 were constructed in this study.

The constructed strains were stored on YPD or YNB medium in a deep freezer at - 80℃ suspended in 15% glycerol.

Table 2. List of the strains used in this study

Name	Description
D452-2	*Saccharomyces cerevisiae* (*Matα, leu2 his3 ura3 can1*)
D_BS6H	D452-2 (pD_BS_6His)
D_EA6H	D452-2 (pD_EA_6His)
SOS4	D452-2 *Δpdc1*, *Δpdc5* (C_2-independent and high glucose-tolerant)
BD_BS	SOS4 (pS_BS, pD_BS and pB_SC)
BD_KP	SOS4 (pS_KP, pD_KP and pB_SC)
BD_KO	SOS4 (pS_KO, pD_KO and pB_SC)
BD_EA	SOS4 (pS_EA, pD_EA and pB_SC)
BD_BS_KP	SOS4 (pS_BS, pD_KP and pB_SC)
BD_BS_KO	SOS4 (pS_BS, pD_KO and pB_SC)
BD_BS_EA	SOS4 (pS_BS, pD_EA and pB_SC)
BD_BS_EA_HGG	SOS4 (pS_BS_H, pD_EA and pB_SC)
BD_BS_EA_GHG	SOS4 (pS_BS′, pD_EA_H and pB_SC)
BD_BS_EA_GGH	SOS4 (pS_BS′, pD_EA and pB_SC_H)
BD_BS_EA_GHH	SOS4 (pS_BS′, pD_EA_H and pB_SC_H)

2.2. Plasmids

Six plasmids were used as mother vectors which have the *GPD* or *HXT7* promoter and *CYC1* terminator from *S. cerevisiae.* (Fig. 5) These are cloning vectors for an episomal expression system of the 2,3-butanediol biosynthetic pathway, *alsS, budB, alsD* and *budA* genes from *B. subtilis*, *K. pneumoniae, K. oxytoca* and *E. aerogenes, BDH1* genes from *S. cerevisiae* (Table 3) There are the oligonucleotide sequence of primers used cloning. (Table 4)

Abbreviations and significations used in this study are as follows. S means α-acetolactate synthase coding gene such as *alsS* or *budB* genes. D means α-acetolactate decarboxylase coding gene such as *alsD* or *budA* genes. B means *BDH1* from *S. cerevisiae*. H is truncated *HXT7* pomoter. KP, KO, EA and BS means derived from *K. pnumoniae*, *K. oxytoca*, *E. aerogenes*, and *B. subtilis* repectively.

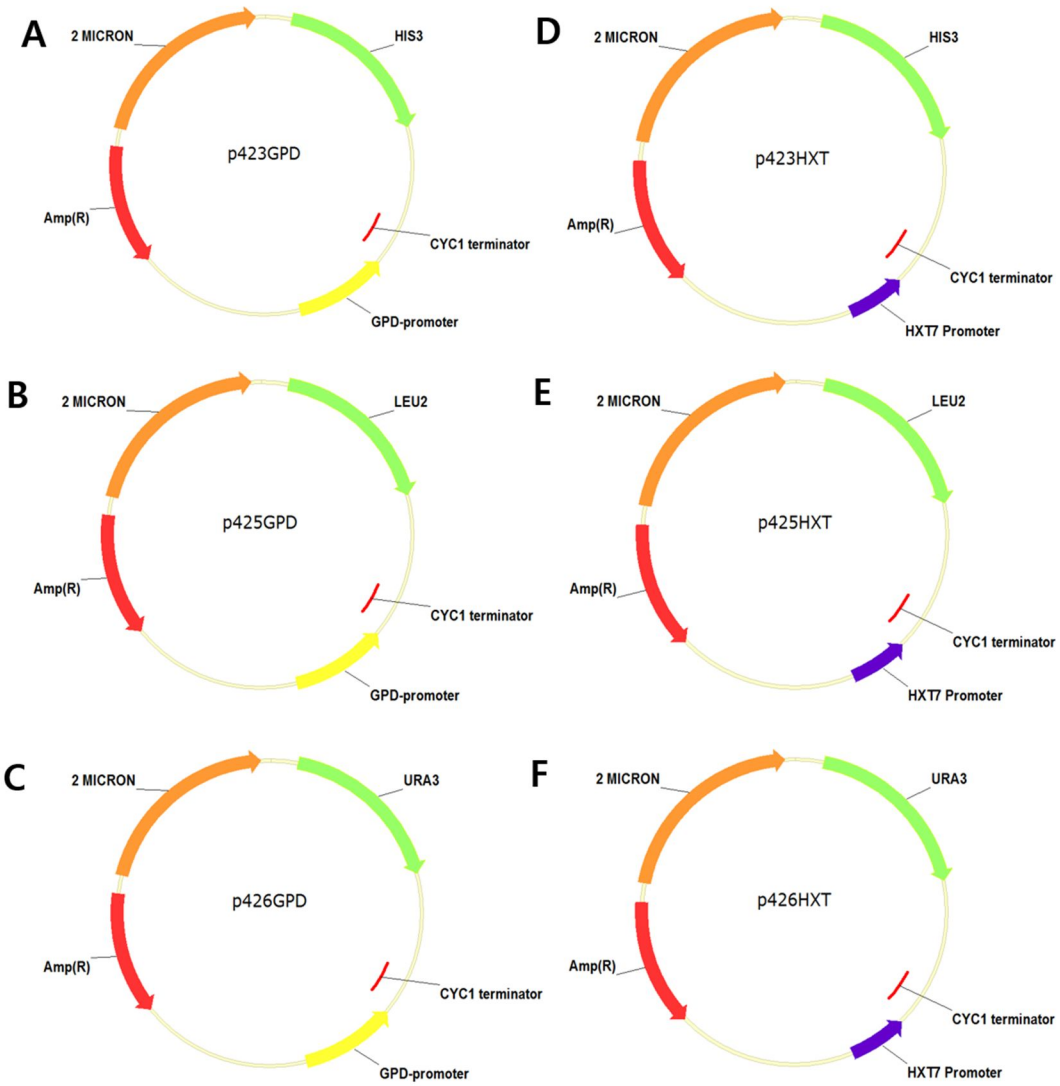

Figure 5. Mother vectors used in this study.

(A) p423GPD, (B) p425GPD, (C) p426GDP,

(D) p423HXT, (E) p425HXT, (F) p426HXT

Table 3. List of the plasmids with *GPD* and *HXT7* promoters used in this study

Name	Description
pRS426GPD	*URA3*, *GPD* promoter, *CYC1* terminator, *2μ* origin, Amp^r
pRS423GPD	*HIS3*, *GPD* promoter, *CYC1* terminator, *2μ* origin, Amp^r
pRS425GPD	*LEU2*, *GPD* promoter, *CYC1* terminator, *2μ* origin, Amp^r
pRS426HXT	*URA3, HXT7* promoter, *CYC1* terminator, *2μ* origin, Amp^r
pRS423HXT	*HIS3, HXT7* promoter, *CYC1* terminator, *2μ* origin, Amp^r
pRS425HXT	*LEU2, HXT7* promoter, *CYC1* terminator, *2μ* origin, Amp^r
pS_KP	pRS423GPD harboring *alsS* gene from *Klebsiella pneumonia* KACC 14816
pS_KO	pRS423GPD harboring *budB* gene from *Klebsiella oxytoca* ATCC 43863
pS_EA	pRS423GPD harboring *budB* gene from *Enterobacter aerogenes* KTCC 13732
pS_BS'	pRS423GPD harboring *alsS* gene from *Bacillus subtilis* str.168
pS_BS_H	pRS423HXT harboring *alsS* gene from *B.*str.168
pD_KP	pRS426GPD harboring *budA* gene from *K. pneumonia* KACC 14816
pD_KO	pRS426GPD harboring *budA* gene *K. oxytoca* ATCC 43863

pD_EA	pRS426GPD harboring *budA* gene from *E. aerogenes* KTCC 13732
pD_EA_H	pRS426HXT harboring *budA* gene from *E. aerogenes* KTCC 13732
pB_SC_H	pRS425HXT harboring *BDH1* gene from *S. cerevisiae* D452-2
pD_BS_6His	pRS423GDP harboring *alsD* gene from *B. subtilis* str.168 and tagging 6 histidine at C-terminal of *alsD* gene
pD_EA_6His	pRS426GDP harboring *budA* gene from *E. aerogenes* KTCC 13732 and tagging 6 histidine at C-terminal of *budA* gene
pS_BS	pRS426GPD harboring *alsS* gene from *B. subtilis* str.168
pD_BS	pRS423GPD harboring *alsD* gene from *B. subtilis* str.168
pB_SC	pRS425GPD harboring *BDH1* gene from *S. cerevisiae* D452-2

pRS423GPD, pRS425GPD and pRS426GPD were donated (Christianson et al., 1992).

pS_BS, pD_BS and pB_SC were constructed by S. J. Kim at Seoul National University in Korea (Kim et al., 2013).

Other plasmids in Table 4 were constructed in this study.

Table 4. List of oligonucleotide used in this study

Primer name	Oligonucleotide sequence (5' → 3')
F_alsS_BamH1_KP	CGCGGATCCAAAATGGACAAACAGTATCCGGTAC
R_alsS_Xho1_KP	CCGCTCGAGTTTACAGAATCTGACTCAGATGCA
F_budB_BamH1_KO	CGCGGATCCAAAATGGTGGATAATCAACATC
R_budB_Xho1_KO	CCGCTCGAGTTTAAAGTATTTGACTGAGATGGAGC
F_budB_Spe1_EA	GGACTAGTAAAATGGACAAACAGTATCCGC
R_budB_Xho1_EA	CCGCTCGAGTTTAAAGAATTTGACTCAGGTGTAGC
F_budA_BamH1_KP	CGCGGATCCAAAATGAATCACTCTGCTGAATGC
R_budA_Xho1_KP	CCGCTCGAGTTTAACTTTCTACGGAACGGAT
F_budA_BamH1_KO	CGCGGATCCAAAATGAACCATTCTGTTGAATGC
R_budA_Xho1_KO	CCGCTCGAGTTTAGTTTTCGACTGAGCGAAT
F_budA_BamH1_EA	CGCGGATCCAAAATGAATCATGCTTCAGATTGC
R_budA_Xho1_EA	CCGCTCGAGTTTAACTTTCTACTGAACGGATGG
R_budA_Xho1_EA_6HIS	CCGCTCGAGTTTAATGATGATGATGATGATGACTTTCTACTGAACGGAT
R_alsD_Xho1_BS_6HIS	CCGCTCGAGTTAATGATGATGATGATGATGTTCAGGGCTTCCTTCAG

3. DNA manipulation and transformation

3.1. Enzymes

Restriction enzymes and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, MA, USA). T4 DNA ligation mix was obtained from Takara (Tokyo, Japan).

3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed with the Accupower™ PCR PreMix (Bioneer Co., Daejon, Korea) in GeneAmp PCR System 2400 (*Applied* Biosystems, CA, USA). PCR solution was composed of 10 pmol of forward and reverse primers, and 10 ng of plasmid DNA as a template. PCR amplification was performed as follows; 1 cycle of 95 °C for 5 min; 30 cycles of 94 °C for 45 sec, 55 °C for 30 sec, 72 °C for 1 min, 1 cycle of 72 °C for 10 min. The amplified gene was confirmed by gel electrophoresis.

3.3. Preparation of plasmid DNA and bacteria genomic DNA

Mini-scale preparation of plasmid DNA was carried out using *Dyne*

^{TM} Plasmid Miniprep Kit from Dyne Bio Co. (Seongnam, Korea) according to the manufacturer's instruction.

Preparation of the genomic DNA to obtain a template for the gene was carried out using using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany) according to the manufacturer's instruction.

3.4. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook (Sambrook et al., 1989). *E. coli* Top10 was cultured in 5 mL LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) for 12hr. 0.5 mL of the culture was transferred to fresh 50 mL LB medium and cultured until OD_{600} reached 0.5. Cells harvested by centrifugation at 6000 rpm for 5 min at 4℃ were resuspended in 5 mL of cold 100 mM $CaCl_2$ solution containing 15 % (v/v) glycerol. Resuspended cells were aliquoted to 100 μL, mixed with DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42℃ for 45 sec, and 1 mL of LB medium was added to the test tubes and incubated at 37℃ for 1 hour to allow the bacteria to express the

antibiotic resistance. Transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.5. Yeast transformation

Transformation of expression vectors was performed using the yeast EZ-Transformation kit (BIO 101, Vista, Calif.). Transformants were selected on YNB medium containing 20 g/L glucose. Amino acids and nucleotides were added as necessary.

4. Media and culture conditions

4.1. Media

LB medium with 50 μg/mL ampicillin was used for recombinant *E. coli* cultivation.

YP medium (1% yeast extract, 2% bacto-peptone) and YNB medium which lacked appropriate amino acid were used for selection of yeast strains. YNB Synthetic Complete medium (6.7 g/L yeast nitrogen base without amino acid, 2.0 g/L amino acids mixture without histidine, tryptophan, leucine or uracil) was used for cultivation of yeast strain.

4.2. Batch fermentations

Batch fermentation was performed in 250 mL flask with 50 mL working volume of YP medium containing 100 g/L glucose at 30 ℃ in shaking incubator (Vision Korea), and shaking rate was maintained at 80 rpm for creating micro-aerobic conditions.

Seed cultures were prepared by culturing in 5 mL test tube of YNB medium containing 20 g/L glucose and incubated overnight at 30 ℃, 250 rpm in shaking incubator (Vision, Korea). Yeast cells were harvested at a mid-exponential phase and inoculated into main cultures with initial OD_{600} of ~1.0.

4.3. Fed-batch fermentations

To obtain a high concentration of 2,3-butanediol, fed-batch fermentation was carried out using a 1Lbench-top fermenter (FERMENTEC, Korea). Fed-batch fermentation was performed in 500 mL of YP medium with 100 g/L glucose at 30℃ and pH 5.5. 200 rpm of agitation speed and 1.0 vvm of aeration were maintained throughout the cultivation.

Seed cultures were prepared by culturing in a 5 mL test tube of

YNB medium containing 20 g/L glucose in a shaking incubator at 30℃ and 250 rpm for 72 h. Pre-cultures were prepared by inoculating the seed cultures in a 500 mL flask with 100 mL working volume of YNB medium containing 20 g/L glucose and grown in a shaking incubator at 30℃ and 250 rpm for 72 h.

Yeast cells were prepared by growing cells overnight to an OD_{600} of 5~10. The cells were harvested by centrifugation at 3000 rpm for 10 min and washed in 5 mL of sterilized DDW and inoculated into the bioreactor with initial OD_{600} of ~10. During the repeated batch fermentation, 100 g/L feeding solution of glucose was intermittently supplied when glucose was depleted.

5. Analysis

5.1. Dry cell weight

Cell growth was monitored by optical density (OD) at 600 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Dry cell weight (DCW) was estimated by using a conversion factor (Oh et al., 2012).

$$\text{Dry cell weight (g/L)} = 0.3 \times OD_{600}$$

5.2. Metabolite detection

Concentrations of glucose, pyruvate, glycerol, acetate, acetoin, 2,3-butanediol and ethanol were measured by a high performance liquid chromatography (Agilent Technologies 1200 Series, U.S.A) with a Bio-Rad Aminex HPX-87H column. The column was eluted with 5mM sulfuric acid at a flow rate of 0.6 ml/min at 60℃. Detection was made with a reflective index detector at 35 °C. Pyruvate was detected by a UV detector at 215 nm.

6. Protein purification

6.1. Sample preparation

For characterization of α-acetolactate decarboxylase (ALDC), D452-2 harboring plasmid pD_BS_6HIS or pD_EA_6HIS was cultured in a 500 mL flask with 200 mL working volume of YNB medium containing 20 g/L glucose and grown in a shaking incubator at 30℃ and 250 rpm for 48 h. After the culture broth was centrifuged at 3000 rpm and 4℃ for 10 min. Discard supernatant and add glass beads, protease inhibitor and His-tag binding buffer [20 mM sodium phosphate (pH 7.4), 500 mM NaCl and 30 mM imidazole]. Glass beads were added 1 g per g of cell weight. Yeast cells were lysed using vortexing for 10 min. To gather expressed protein, Yeast cells were centrifuged at 3000 rpm and 4℃ for 10 min. The supernatant was mixed with a His-tag binding buffer.

6.2. Affinity chromatography

The prepared samples were loaded into the Poly-Prep Chromatography Columns (Bio-rad) with 0.5 mL $Ni2^{+}$-NAT His-Bind resin (Qiagen). After equilibrating the column with binding

buffer, the sample resuspended in binding buffer was loaded. The column loaded with the sample was washed with binding buffer, and bound proteins were eluted with elution buffer [20 mM sodium phosphate (pH 7.4), 500 mM NaCl and 500 mM imidazole]. The size of purified proteins was analyzed by SDS-PAGE (Mallu et al., 2014)

7. α-Acetolactate decarboxylase activity assay

7.1. Enzyme activity assay

Ethyl 2-acetoxy-2-methylacetoacetate (diester of α-acetolactate) is transformed into α-acetolactate, ethanol and acetate by saponification. Ethy 2-acetoxy-2-methylacetoacetate is added to 180 mM sodium hydroxide at 10℃ for 20 min in a stirred closed vessel. Then, 100 mM maleate buffer (pH 6.0) and 100 mM maleic acid are added. The obtained solution corresponds to 15.4 mM α-acetolactate in maleate buffer at pH 6.0. It has to be prepared just before use and to be used within the following 3 h.

The assay is performed at 10℃ in a controlled temperature room. α-acetolactate decarboxylase solution are poured into capped tubes disposed on a shaking device at 10℃. The reaction is allowed to

occur by addition of 15 mM α-acetolactate solution under stirring. After 20 min, the enzymatic reaction is stopped by addition of an equivalent volume of 2.5 M sodium hydroxide and vigorous mixing. Acetoin production is determined by the method described below and activity is expressed as molar concentration of acetoin formed per minute (Dulieu et al., 1999)

The determination of acetoin concentration is carried out by a colorimetric method. Acetoin is reacted with guanido groups of creatine in alkaline medium to give pink color. A color-reagent is prepared by mixing an equal volume of 0.2% creatine solution in distilled water and 2% α-naphthol freshly dissolved in 2.5 M sodium hydroxide. This color-reagent must be protected from light and used within 4 h. A color-reagent is added with sample medium in eppen tube, mixed, and left at 20℃. After exactly 40 min of color development, samples are introduced into a spectrophotometric 96-well microplate and red absorbance at 525 nm (Dulieu et al., 1999).

To characterize the enzymatic properties of ALDCs, his-tagging ALDCs purified as described above were used. Using initial acetoin concentrations of 0.91, 1.82, 2.73, 3.64, 4.55, and 5.45 mM, kinetic constants of kcat, Km, and kcat/Km were obtained from each

Lineweaver-Burk plot. Standards of acetoin (0.27 mM to 1.08 mM in same step and solution described above) are analyzed at the beginning and at the end of each set of measurements.

7.2. Bradford assay

BSA (Bovine Serum Albumin) standard protein was prepared to determine a standard curve for quantitative analysis of ALDC. BSA with concentrations of 0, 5, 10, 15 μg/mL for the standard assay was used. ALDC was diluted with distilled water. 200 μl of Quick Start Bradford protein 1x dye reagent from Quick Start Bradford Assay Kit and 800 μl of protein were combined and vortexed. Combined samples were incubated at room temperature for 5 minutes, and measured absorbance at 595 nm.

III. RESULTS AND DISCUSSIONS

1. Construction of the efficient 2,3-butanediol biosynthetic system

1.1. Investigation of 2,3-butanediol biosynthetic genes from various microorganisms

In the previous study by Dr. S.J. Kim, introducing the *alsS* and *alsD* genes from *B. subtilis* and overexpressing the endogenous *BDH1* gene in pyruvate decarboxylase-deficient *S. cerevisiae* (SOS4) were performed. ALS and ALDC genes from various microorganisms were investigated to search for improving 2,3-butanediol production. Therefore, three microorganisms, *K. pnuemoniae, K. oxytoca*, and *E. aeregenes*, producing 2,3-butanediol more than 100 g/L were selected. Introducing ALS and ALDC from above three microorganisms and overexpressing the endogenous *BDH1* gene in the SOS4 strain for 2,3-butanediol biosynthetic pathway were constructed.

BD_KP, BD_KO and BD_EA strain were constructed using above plasmids and the control strain was BD_BS strain. Batch fermentation profiles of the constructed strains are displayed in

Figure 6.

While BD_KP, BD_KO, and BD_EA produced 21.9 g/L, 18.9 g/L, and 21.6 g/L of 2,3-butanediol respectively, BD_BS produced 29.3 g/L of 2,3-butanediol from 100 g/L of glucose within 168 h under micro-aerobic conditions without production ethanol. The control strain, BD_BS strain, still produced 2,3-butanediol more than other strains. Also BD_BS strain less accumulate pyruvate which is important substrate in the 2,3-butanediol production (Table 5). Because ALS from *B. subtilis* has stronger preference for pyruvate to 2-ketobutyrate than ALSs from other microorganisms (Gollop et al., 1990). To less accumulate pyruvate and improve 2,3-butanediol production, ALS of *B. subtilis* was continuously used.

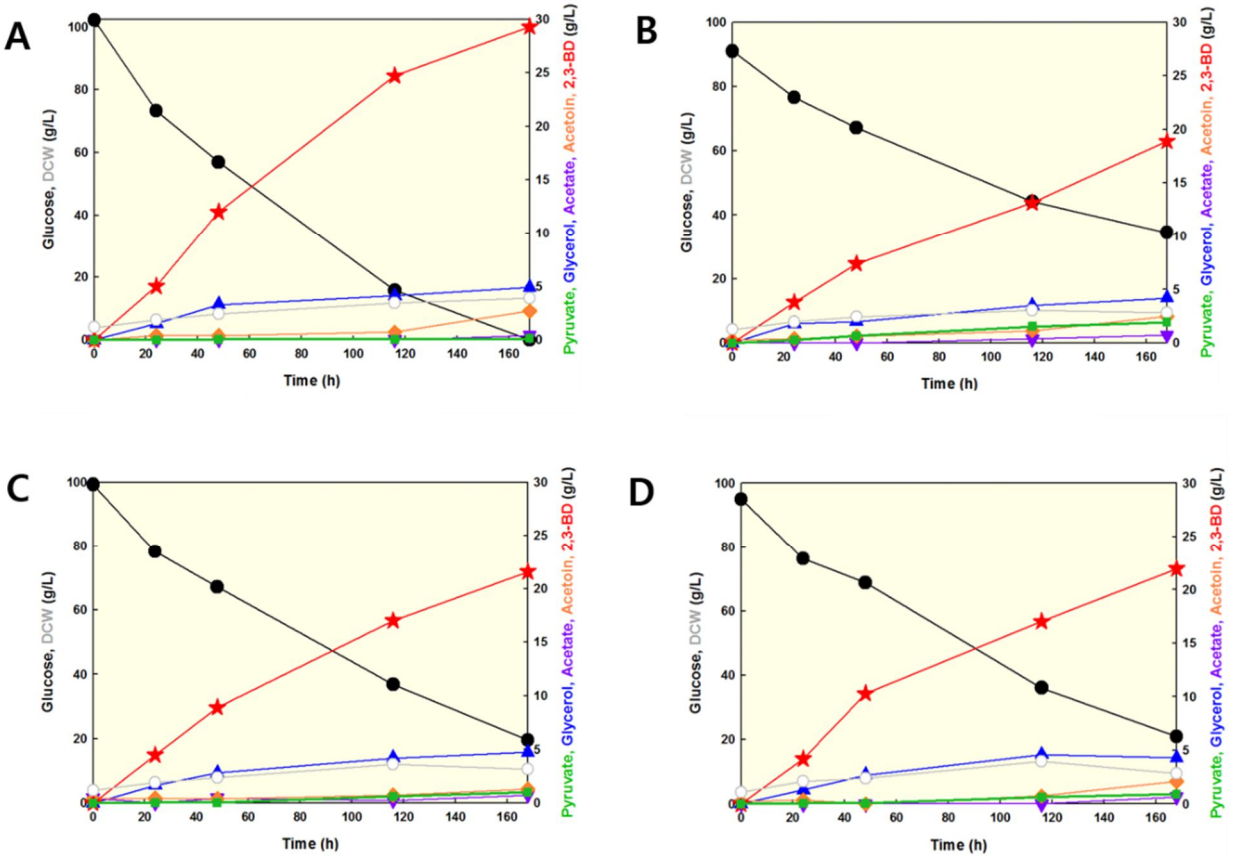

Figure 6. Batch fermentations by SOS4 strain with ALS and ALDC genes from various microorganisms.

(A) BD_BS, (B) BD_KP, (C) BD_KO, (D) BD_EA

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Acetoin ( ), 2,3-Butanediol ( )

Table 5. Summary of batch fermentations in part 1.1

Strain	DCW (g/L)	Glucose consumption rate (g/L·h)	Pyruvate (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	2,3- BD Yield (g/g)	2,3-BD productivity (g/L·h)
BD_BS	13.4	0.61	0.14	4.90	2.70	29.29	0.29	0.17
BD_KP	9.48	0.44	0.90	4.30	2.10	21.94	0.23	0.13
BD_KO	9.45	0.34	1.94	4.20	2.49	18.90	0.21	0.11
BD_EA	10.6	0.47	1.01	4.72	1.29	21.57	0.22	0.13

1.2. Enhancement of 2,3-butanediol production by combination of ALS

According to the previous results, BD_BS_KP, BD_BS_KO, and BD_BS_EA strains were constructed by combination of ALS from *B. subtilis* and ALDCs from *K. pnuemoniae, K. oxytoca*, and *E. aerogenes* in the SOS4 strain. Additionally, the endogenous *BDH1* gene was overexpressed to enforce the acetoin conversion into 2,3-butanediol. Batch fermentation profiles of the constructed strains are displayed in Figure 7.

The control strain, BD_BS strain, produced 28.8 g/L 2,3-butanediol from initial 100 g/L glucose within 168 h under micro-aerobic conditions without production ethanol. Whereas the BD_BS_KP, BD_BS_KO, and BD_BS_EA strain produced 23.7,g/L, 22.5 g/L, and 34 g/L 2,3-butanediol respectively (Table 6).

The BD_BS_EA strain obtained higher 2,3-butanediol yield (0.34 g 2,3-butanediol/g glucose) than the control strain, BD_BS strain (0.31 g 2,3-butanediol/g glucose). These results suggested that the metabolic fluxes from pyruvate toward 2,3-butanediol in the BD_BS_EA strain could be higher than the control strain. So the BD_BS_EA strain more rapidly consumed glucose than the control strain. However, to inquiry the potential ability of 2,3-butanediol

production between the BD_BS strain and the BD_BS_EA strain as a 2,3-butanediol producer, fed-batch fermentations need to carried out through intermittent addition of glucose under the optimum aeration condition.

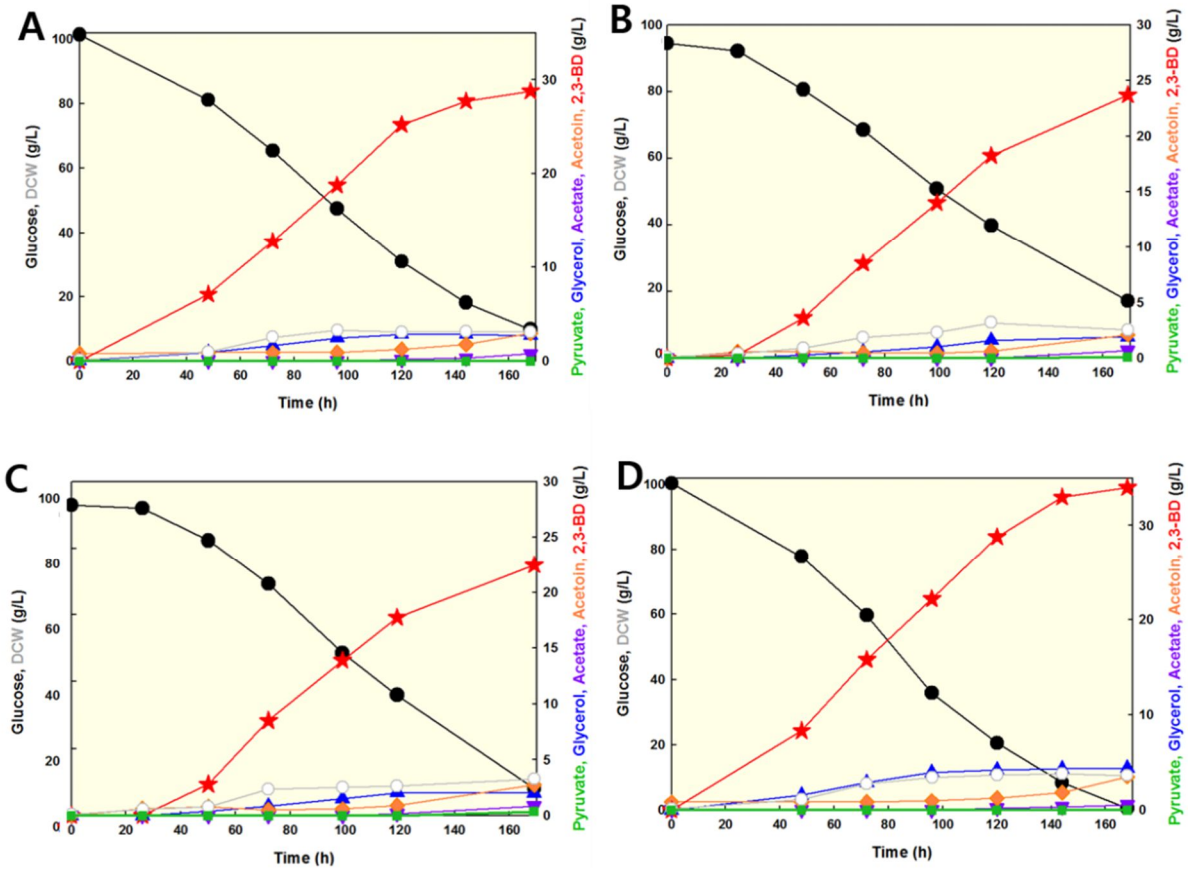

Figure 7. Batch fermentations by the SOS4 strain with combination of ALS from *B. subtilis* and ALDCs from various microorganisms.

(A) BD_BS, (B) BD_BS_KP, (C) BD_BS_KO, (D) BD_BS_EA

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Acetoin ( ), 2,3-Butanediol ( )

Table 6. Summary of batch fermentations in part 1.2

Strain	DCW (g/L)	Glucose consumption rate (g/L·h)	Pyruvate (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	2,3- BD Yield (g/g)	2,3-BD productivity (g/L·h)
BD_BS	9.1	0.55	0.0	2.7	3.0	28.8	0.31	0.17
BD_BS_KP	8.4	0.46	0.1	1.9	2.1	23.7	0.31	0.14
BD_BS_KO	11.0	0.50	0.3	2.0	2.7	22.5	0.26	0.13
BD_BS_EA	10.7	0.59	0.0	4.4	3.5	34.0	0.34	0.20

2. Promoter replacement to increase 2,3-butanediol biosynthetic genes expression level

In previous parts of this study, all genes were controlled under the *GPD* promoter. To enhance the 2,3-butanediol biosynthetic pathway, the *GPD* promoter of *alsS, budA*, and *BDH1* gene of the BD_BS_EA strain was replaced with the truncated *HXT7* promoter. These constitutive promoters are normally used for overexpression of heterologous genes in yeast. Particularly, the truncated *HXT7* promoter is known as 30 ~ 40 % stronger than *GPD* promoter in yeast (Hauf et al., 2000).

BD_BS_EA_HGG, BD_BS_EA_GHG, BD_BS_EA_GGH, and BD_BS_EA_GHH were constructed using plasmids containing the truncated *HXT7* promoter and cultured in YP medium with 100 g/L glucose under micro-aerobic conditions. Batch fermentation profiles of the constructed strains are displayed in Figure 8. As the expression levels of promoter was changed, yield of 2,3-butanediol was not better improved than the control strain, BD_BS_EA_GGG (Table 7).

In this case, stronger promoter expression of the *alsS, budA*, and

BDH1 gene of the BD_BS_EA strain might reduce ability to produce 2,3-butanediol. Among the promoter tested, in this thesis, expression of the *alsS, budA*, and *BDH1* gene of the BD_BS_EA strain under control of the *GPD* promoter was continuously used for 2,3-butanediol production.

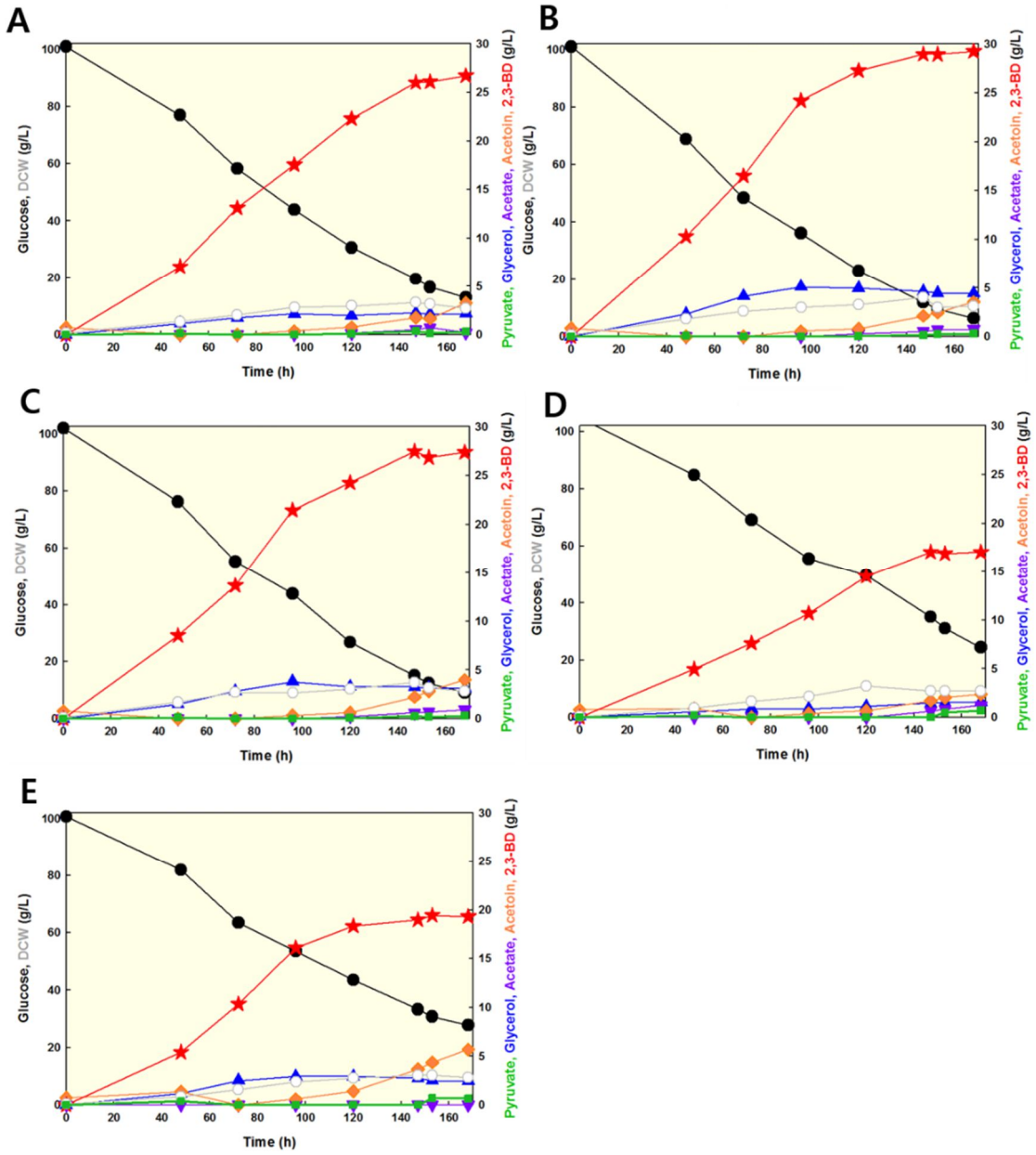

Figure 8. Batch fermentations by the BD_BS_EA strains with different strength promoters

(A) BD_BS_EA_GGG, (B) BD_BS_EA_HGG, (C) BD_BS_EA_GHG

(D) BD_BS_EA_GGH, (E) BD_BS_EA_GHH

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ),

Acetate ( ), Acetoin ( ), 2,3-Butanediol ( )

Table 7. Summary of batch fermentations in part 2

Strain	DCW (g/L)	Glucose consumption rate (g/L·h)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	2,3- BD Yield (g/g)	2,3-BD productivity (g/L·h)
BD_BS_EA_GGG	9.27	0.52	2.16	3.27	26.71	0.304	0.16
BD_BS_EA_HGG	10.60	0.56	4.41	3.53	29.23	0.309	0.17
BD_BS_EA_GHG	9.63	0.55	3.10	3.99	27.37	0.295	0.16
BD_BS_EA_GGH	9.27	0.48	1.56	2.34	17.04	0.212	0.10
BD_BS_EA_GHH	9.60	0.43	2.44	5.68	19.3	0.265	0.11

3. 2,3-Butanediol production by the BD_BS_EA strain in fed-batch fermentation

To inquiry the potential ability of 2,3-butanediol production between the BD_BS strain and the BD_BS_EA strain as a 2,3-butanediol producer, fed-batch fermentations were carried out through intermittent addition of glucose under the optimum aeration condition using bioreactor. The fed-batch fermentation profiles of the BD_BS_EA and BD_BS strains are displayed in Figure 9.

The final concentration of 2,3-butanediol of the BD_BS_EA strain was 132.4 g/L after 322 h cultivation, with a 2,3-butanediol yield (0.34 g 2,3-butanediol/g glucose) and productivity (0.41 g 2,3-butanediol/L·h). The BD_BS_EA strain yielded higher 2,3-butanediol yield and productivity than the control strain, BD_BS strain, by 28% and 24% respectively (Table 8). As fermentation time passed, in the BD_BS strain, the carbon fluxes were probably moved toward not 2,3-butanediol biosynthesis but respiration to maintain cell living. Also, in the BD_BS_EA strain, because the 2,3-butanediol biosynthesis pathway be intensified, it can be seen that the glycerol yield was more increased than the control strain. It was

assumed that other unknown pathway was detoured to 2,3-butanediol biosynthesis pathway.

To reveal that BD_BS_EA strain was obtained the highest 2,3-butanediol yield than the control strain, specific activity of *budA* gene from *E. aerogenes* should be measured in *S. cerevisiae*.

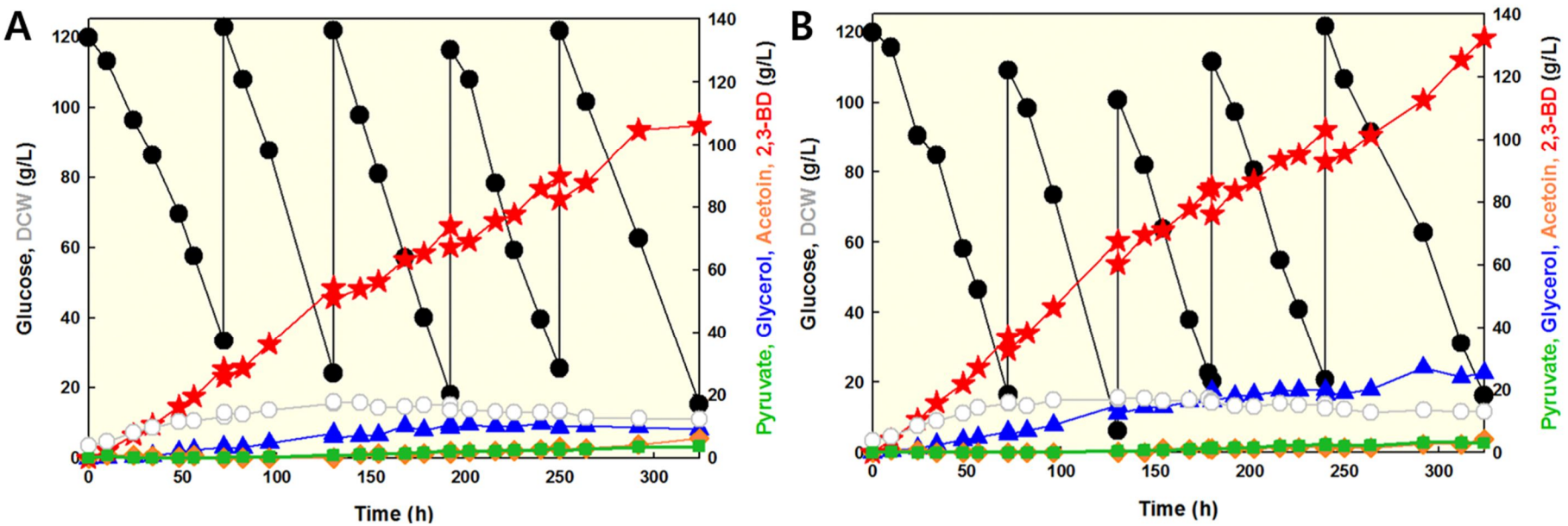

Figure 9. Fed-batch fermentations.

(A) BD_BS, (B) BD_BS_EA

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ),

Acetate ( ), Acetoin ( ), 2,3-Butanediol ( )

Table 8. Summary of fed-batch fermentations

Strain	DCW (g/L)	Glucose consumption rate (g/L·h)	Pyruvate (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	Glycerol Yield (g/g)	2,3-BD Yield (g/g)	2,3-BD productivity (g/L·h)
BD_BS	11.0	1.50	3.7	9.1	6.2	106.4	0.03	0.26	0.33
BD_BS_EA	11.6	1.49	3.0	25.3	4.2	132.4	0.07	0.34	0.41

4. Specific activity assay and Kinetic analysis of α-acetolactate decarboxylase

To analyze the kinetic properties of ALDC from *B. subtilis* and *E. aerogenes*, ALDC purification was carried out by using affinity chromathography. Plasmids that six histidine residues were tagged at the C-terminal of ALDC genes were constructed. D_BS6H and D_EA6H strain were constructed by introducing those plasmids into *S. cerevisiae* D452-2. SDS-PAGE analysis indicated that the target protein was purified and detected at the size of molecular weight of ALDC (Fig. 10)

After purification by the His-tag system, the purified ALDC from the control (*B. subtilis*) and *E. aerogenes* were subjected to determination of kinetic constants and specific activity. As shown in Figure 11, while specific activity of ALDC in the control was 106 mU/mg protein, ALDC in *E. aerogenes* was 250.4 mU/mg protein. It was about 2-folds higher than the control. Although ALDC from the control possessed a higher substrate affinity (*Km*), a rate constant (*kcat*) was much lower than ALDC from *E. aerogenes*. So, *kcat*/*Km* value of ALDC from *E. aerogenes* was 40% higher than the control

(Table 9). However substrate (α-acetolactate) affinity of ALDC from *E. aerogenes* was lower, the product (acetoin) conversion efficiency was higher than the control. Therefore, because overall activity of 2,3-butanediol production by the BD_BS_EA strain was increased, 2,3-butanediol yield was much more increased than the control strain.

In this study, the highest 2,3-butanediol concentration from glucose as high as 2,3-butanediol production by native bacteria was obtained by introducing ALS from *B. subtilis* and ALDC from *E. aerogenes*, and overexpressing *BDH1* into Pdc-deficient *S. cerevisiae* (SOS4).

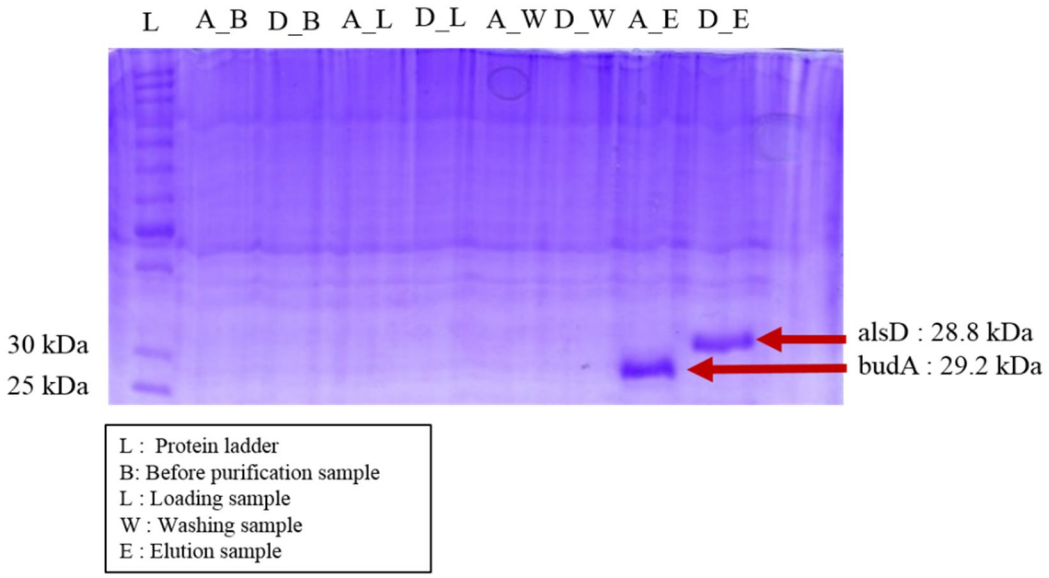

Figure 10. SDS-PAGE analysis of α-Acetolactate decarboxylase purification by His-tag system. Samples of purification steps were used. *alsD* from *B. subtilis* and *budA* from *E. aerogenes*

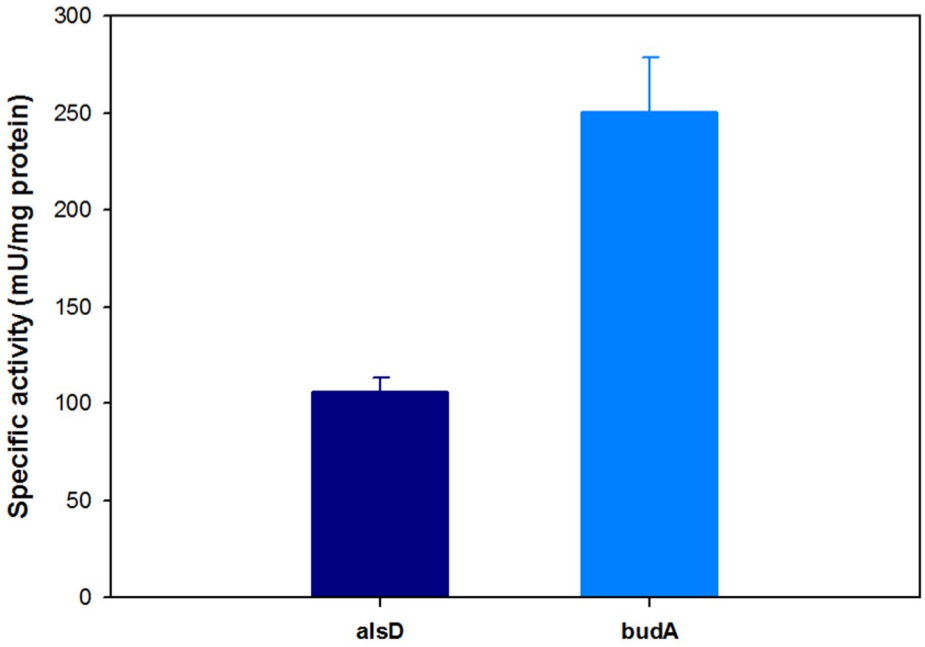

Figure 11. Specific activity of α-acetolactate decarboxylase.

alsD from *B. subtilis* and *budA* from *E. aerogenes*

Table 9. Kinetic parameters of purified α-acetolactate decarboxylase

Gene	Origin	Specific activity (mU/mg)	Vmax (μM/min)	*Km* (mM)	*kcat* (s^{-1})	*kcat*/*Km* ($mM^{-1} \cdot s^{-1}$)
alsD	*B. subtilis*	106.0 ± 7.4	4.9 ± 0.5	0.6 ± 0.3	9.5 ± 0.9	17.3 ± 7.6
budA	*E. aeregenes*	250.4 ± 28.5	26.6 ± 2.1	1.1 ± 0.2	26.2 ± 2.1	24.6 ± 3.0

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) The Pdc-deficient *S. cerevisiae* harboring the *alsS* gene from *B. subtilis*, the *budA* gene from *E. aerogenes* and the *BDH1* gene from *S. cerevisiae* (BD_BS_EA strain) was the best for production of 2,3-butanediol in terms of yield and productivity of 2,3-butanediol

(2) The BD_BS_EA strain produced 132.4 g/L of 2,3-butanediol from glucose with a 2,3-butanediol yield (0.34 g 2,3-butanediol/g glucose) and productivity (0.41 g 2,3-butanediol/L·h) in fed-batch fermentation.

(3) To confirm a reason for such an increase in 2,3-butanediol yield of the BD_BS_EA strain, the kinetic properties of ALDC were analyzed. The specific activity and *kcat*/*Km* value of α-acetolactate decarboxylase isolated from *E. aerogenes* were 250 mU/mg protein, 24.6 $mM^{-1} \cdot s^{-1}$ respectively.

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국 문 초 록

2,3-Butanediol (2,3-BD)는 산업적 활용도가 높은 화학 소재이다. 2,3-BD은 화학적 생산이 가능하지만 유가 변동문제로 인해 최근 바이오 기술이 개발됨에 따라 생물공학적으로 생산이 주목 받고 있다. 생물공학적 생산에는 주로 박테리아가 사용되는데 고수율로 2,3-BD를 생산할 수 있지만 이들 대부분이 병원성 박테리아로 분류된다. 식품 첨가물과 화장품 원료의 전구체로 이용되는 2,3-BD를 박테리아로 생산하게 되면 안전과 산업화 측면에서 대량생산공정 구축이 어려워진다. 그 대안으로 GRAS (Generally Recognized As Safety)미생물로서 안전하다고 알려진 *Saccharomyces cerevisiae*를 이용한 2,3-BD 생산에 주목할 필요가 있다. 하지만 *S. cerevisiae*는 자연상태에서 2,3-BD를 거의 생산하지 못하고 에탄올을 주로 생산하기에 고효율의 2,3-BD를 생산하기 위해서는 대사공학적 방법을 이용한 재조합 *S. cerevisiae*의 구축이 요구된다.

따라서 선행연구자에 의해 *S.* cerevisiae 의 주요 대사 산물인 에탄올 생성을 억제하고자 pyruvate decarboxylase 활성이 완전히 저해된 효모(SOS4)를 제작한 바 있다. 이렇게 2,3-BD의 주요 전구체인 파이루베이트를 축적하는 SOS4 균주에 2,3-BD 생합성 경로를 강화시켰지만 여전히 박테리아 수준만큼의 2,3-BD 농도와 수율 정도가 미약하여 이를 개선할 필요가 있다.

본 연구에서는 새로운 2,3-BD에 관련된 유전자를 찾아 기존보다 더 높은 생산 수율을 얻는 것이 최종목표이다. 첫 번째로 2,3-BD를 고수율로 생산하는 박테리아들을 조사하였고, 약 100 g/L가 넘게 2,3-BD를 생산하는 *Klebsiella pneumoniae*, *K. oxytoca* 그리고 *Enterobacter aerogenes* 총 3가지를 선택하였다. 따라서 이들 균들의 acetolactate synthase(ALS)를 암호화하는 유전자와 acetolactate decarboxylase(ALDC)를 암호화하는 유전자를 효모에 도입시켰고, 추가적으로 *S. cerevisiae* 내부의 *BDH1* 유전자를 과발현 시켜 균주를 구축하였다. 하지만

이들을 플라스크 수준에서 발효를 진행했을 때 대조군인 *Bacillus subtilis* 유래의 유전자가 도입된 균주(BD_BS)보다 높은 2,3-BD 수율을 얻을 수 없었다.

두 번째로 파이루베이트가 가장 적게 쌓였던 *B. subtilis* 유래의 ALS를 암호화하는 *alsS* 유전자를 고정시키고, ALDC를 암호화하는 유전자를 3가지 박테리아별로 도입시키고 추가적으로 *BDH1* 유전자를 과발현 시켜 균주를 구축하였다. 이들을 가지고 플라스크 수준에서 발효를 진행하였고, 이 때 *E. aerogenes* 유래의 ALDC를 암호화하는 유전자인 *budA*를 사용한 균주(BD_BS_EA)가 BD_BS 균주보다 2,3-BD 수율이 약 10% 증가한 수치를 보였다. 이 두 균주들의 정확한 발효능력을 보기 위하여 fed-batch fermentation을 수행하였고, BD_BS_EA 균주가 포도당으로부터 약 132 g/L의 2,3-BD를 생산하였으며 수율과 생산성이 각각 0.34 g 2,3-BD/g glucose, 0.41 g/L·h로 생산하였다. 이는 대조군보다 약 28%의 수율과 24%의 생산성이 증가한 수치를 보였다. 이 두 균주의 수율이 왜 차이를 알

아보기 위해 차이점인 ALDC의 activity를 측정하고자 하였다. 각 ALDC의 유전자를 Histidine 부착 방법을 통한 affinity chromatography를 이용하여 단백질을 정제하였다. ALDC의 기질인 α-acetolactate를 이용하여 specific activity를 측정하였다. 그 결과 *E. aerogenes* 유래의 ALDC가 250 mU/mg로 대조군보다 약 2.5배의 activity가 증가한 수치를 보였다. 비록 E. aerogenes 유래의 ALDC가 낮은 기질 친화성을 보였지만, 최대 반응 속도(Vmax)와 기질 변환 효율 (Kcat/Km)이 대조군에 비해 각각 약 5배, 1.5배가 증가한 수치를 보였다. 이를 통하여 재조합 효모인 BD_BS_EA 균주가 2,3-BD를 박테리아만큼 산업적으로 고농도 생산을 위한 안전성이 갖춰진 균주로서 가치 있음을 증명하였다.

주요어 : 2,3-Butanediol (2,3-BD), pyruvate decarboxylase 결여 효모, 유가식 배양, α-acetolactate decarboxylase

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