A Thesis for the Degree of Master of Science

Production of meso-2,3-butanediol in engineered *Saccharomyces cerevisiae*

대사공학적으로 설계된 효모로부터
meso-2,3-butanediol 생산

By

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Department of Agricultural Biotechnology
Seoul National University
February 2019

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재조합 효모를 이용한 meso-2,3-butanediol 생산

指導敎授 徐 鎭 浩

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ABSTRACT

Global warming is an environmental issue at a national level. However, carbon dioxide emissions are still increasing with the use of fossil fuels.

Therefore, the biomass-based systems have been attracting attention to replace the petroleum-based systems. This has an advantage of being carbon neutral because the carbon dioxide generated is absorbed again by biomass.

In this study, the biomass-based microbial factory technology was constructed to produce bio-chemicals. Among various candidates for meso-2,3-butanediol (meso-2,3-BD) has valuable properties as a cosmetic additive and crop protectant.

Most microbial fermentations for meso-2,3-BD production have been focused on bacterial based process, however, most of them are pathogenic and they are liable to be contaminated with other germs such as bacteriophages. This makes difficult large-scale fermentations in

terms of safety and industrialization. As an alternative, *Saccharomyces cerevisiae* is a GRAS (Generally Regarded As Safe) microorganism and has proven its industrial value as being used for commercial ethanol fermentation.

However, a wild type *S. cerevisiae* synthesizes a small amount of R,R-2,3-BD not meso-2,3-BD. In previous studies, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* with the R,R-2,3-BD synthetic pathway was constructed (Kim et al., 2017). In this thesis, the R,R-2,3-BD biosynthetic pathway was eliminated by the CRISPR-Cas9 gene editing system and the pathway for producing meso-2,3-BD was constructed by overexpressing acetoin reductase (*budC*) from *Klebsiella oxytoca*. Finally, the engineered strain produced meso-2,3-BD without producing R,R-2,3-BD.

More research efforts were made in order to improve the yield and productivity of meso-2,3-BD production. First, Pdc-deficient *S.*

cerevisiae requires a trace amount of C_2-compound such as ethanol for cell growth. The previous research found that Pdc-deficient *S. cerevisiae* expressing the *Candida tropicalis PDC1* gene (*CtPDC1*) represented an improvement of the tolerance in non-ethanol supplemental medium (Kim et al., 2016). In this study, the *CtPDC1* gene was integrated into the chromosome with a weak constitutive promoter, GPD2 promoter, by CRISPR-Cas9. With expression of the *CtPDC1 gene*, biomass yield of 2.64 g DCW/ g glucose and a 2,3-BD titer of 24.0 g/L were obtained. The productivity of meso-2,3-BD was 0.21 g/L/h which was 2.3 folds higher than that of the control (0.09 g/L/h). The S5 dG dB::G2Ct SDNC strain produced 131 g/L of meso-2,3-BD with 1.64 g/L/h of productivity and 0.47 g/g of yield in the fed-batch fermentation without ethanol supplement.

Second, the *budC* gene were expressed additionally by using a strong constitutive promoter to further improve yield and productivity for meso-

2,3-BD produce and to reduce acetoin as a by-product. The engineered strain S5 dG::T3bC dB::G2Ct SDNC resulted in an improvement of both yield and productivity. For the fed-batch fermentation with the engineered strain, 171 g/L meso-2,3-BD was produced in 93 h cultivation. The yield of meso-2,3-BD (0.49 g/g) was corresponded to 98 % of the theoretical yield.

The engineered *S. cerevisiae* strain constructed in this study successfully produced meso-2,3-BD with high yield and productivity.

Keywords : meso-2,3-butanediol, pyruvate decarboxylase (Pdc), acetoin reductase, *Saccharomyces cerevisiae*, fed-batch fermentation

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I. INTRODUCTION

1. Meso-2,3-butanediol

2,3-Butanediol (2,3-BD) is a chiral four-carbon compound with the chemical formula of CH3CH(OH)CH(OH)CH3 and its molecular weight of 90.12 kDa. It has three stereoisomeric forms: D-(-)-, L-(+)- and meso. It is known as 2,3-butylene glycol, 2,3-dihydroxybutane, or dimethylethlene glycol (Syu et al., 2001).

Through various chemical reactions such as dehydrogenation, ketalization, esterification, and dehydration, 2,3-BD can be converted into other valuable chemicals such as acetoin and diacetyl which can be used as flavoring agents, cosmetic and food products (Bartowsky and Henschke, 2004; Soltys et al., 2001).

Especially, meso-2,3-BD has an excellent antibacterial activity and can be used as preservatives. It has a possibility to be used as cosmetic additives and crop protectant (Baek et al., 2014).

2. Stereoisomers of 2,3-butnaediol

There are three stereoisomeric forms of 2,3-BD : levo-[D-(−)-, (*R*,*R*)-], dextro-[L-(+)-, (*S*,*S*)-], and meso-forms. The levo- and dextro-forms are optically active, whereas the meso- form shows optically inactive (Syu, 2001). Because it is complicated and expensive to produce pure stereoisomer forms by chemical processes, a biological route by microbial fermentations is preferable to obtain stereoisomers of 2,3-BD selectively (Liu et al., 2011; Ui et al., 2004). It depends on microorganisms to produce each of stereoisomers, and a mixture of two stereoisomers is generally formed (Maddox, 1996). For example, *Klebsiella* sp. and *Enterobacter aerogens* produce meso-2,3-BD and (*S*,*S*)-2,3-BD. *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* also produce a mixture of (*R*,*R*)-2,3-BD and meso-2,3-BD (Celinska and Grajek, 2009). On the other hand, *Paenibacillus polymyxa* and *Serratia marcescens* produce (*R*,*R*)-2,3-BD and meso-2,3-BD in the pure form, respectively. (Ji et al., 2011; Zhang et al., 2010b). Until now, the mechanisms underlying the formation of stereoisomers were not fully understood (Ji et al., 2011).

3. meso-2,3-Butanediol production by microorganisms

A number of bacteria can produce 2,3-BD, but only a few might be regarded as 2,3-BD producers (Garg et al., 1995). Until now, *K. pneumoniae*, *K. oxytoca* and *P. polymyxa* are unbeatable in the efficient production of 2,3-BD with high yield and productivity. Also, *E. aerogenes* and *S. marcescens* are considered promising microorganisms (Ji *et al.* 2011; Celinska *et al.* 2009).

In bacteria, 2,3-BD is produced via a mixed acid fermentation along with by-products such as ethanol, acetate, lactate, formate and succinate (Magee et al., 1987; Maddox et al., 1996). Four key enzymes are involved in 2,3-BD production from pyruvate. Pyruvate from glycolysis 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 present, α–acetolactate can undergo spontaneous decarboxylation producing diacetyl. Then, diacetyl reductase (DAR) can catalyze diacetyl to acetoin. Finally, acetoin can be converted to 2,3-BD by acetoin reductase (AR), also known as butanediol dehydrogenase (BDH) (Celinska et al., 2009).

However, most bacteria for 2,3-BD production are classified as pathogenic microbes (Class II). Therefore, safe microorganisms are

required to be used in order to substitute for these pathogenic 2,3-BD producing bacteria. (Kim et al., 2013; Li et al., 2013).

4. Production of 2,3-BD from pyruvate decarboxylase-deficient *S. cerevisiae*

Traditionally, *S. cerevisiae* is used for fermentation a sugar to ethanol. It is suitable as a host for the production of therapeutic proteins or bio-based chemicals by metabolic engineering. In *S. cerevisiae*, it is necessary to redirect carbon fluxes away from the ethanol synthesis pathway. To maximize the production of desired products, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* has been studied as the host strain for production of lactate, malate and glycerol (Geertman et al., 2006 ; Ishida et al., 2006 ; Zelle et al., 2008)

Pyruvate is located at the branch point between the fermentative and respiratory metabolism. Especially *PDC* converts pyruvate to acetaldehyde, which is further reduced into ethanol by alcohol dehydrogenase. There are three structural genes (*PDC1, PDC5*, and *PDC6*) encoding active pyruvate decarboxylase isoenzymes in *S. cerevisiae*. Disruption of *PDC1* and *PDC5* or all *PDC* genes led to elimination of pyruvate decarboxylase activity completely (Flikweert et al, 1996).

There are defects of Pdc-deficient strains for industrial scale

fermentations. First of all, the Pdc-deficient mutants have two-carbon dependent cell growth pattern. In the other words, 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 through acetate in *S. cerevisiae*. In case of the elimination of Pdc activity, acetaldehyde cannot be produced which leads to the shortage of cytosolic acetyl-CoA. Second of all, the Pdc-deficient mutants showed lower growth rate in a glucose-containing medium than the wild type of *S. cerevisiae*. The Pdc-deficient strains suffer oxidative stress because of redox imbalance with repression of respiration by glucose.

In the previous study, the *PDC1, PDC5* and *PDC6* genes were deleted in the *S. cerevisiae* D452-2 strain (SOS5). To produce 2,3-BD, the *alsS* and *alsD* genes coding for α–acetolactate synthase and α–acetolactate decarboxylase from *B. subtilis* were introduced in the engineered *S. cerevisiae* strain. Additionally, the endogenous *BDH1* gene coding for 2,3-BD dehydrogenase was overexpressed. The resulting strain (BD5) produced 2,3-BD from glucose without ethanol production. However, substantial amounts of glycerol were produced in

parallel with 2,3-BD formation because of redox imbalance by excessive cytosolic NADH under oxygen-limited conditions. To overcome this limitation, expression of *Lactococcus lactis* NADH oxidase in the 2,3-BD-producing Pdc-deficient *S. cerevisiae* reported reducing glycerol while increasing 2,3-BD production (Kim et al., 2015). Expression of the NADH oxidase results in decreasing intracellular NADH concentration and NADH/NAD^+ ratio and reducing glycerol accumulation in the Pdc-deficient *S. cerevisiae*.

Also, a research was reported about a solution of the defect of two-carbon compound dependent, recently. The expression of the *PDC1* gene from *Candida tropicalis* with a fine-tuned expression level led to be a two-carbon compound independent Pdc-deficient *S. cerevisiae*. And the engineered strain(BD5_Ctnox) produced 154.3 g/L of R,R-2,3-BD with 1.98 g/L/h of productivity and 0.404 g/g of R,R-2,3-BD (Kim et al., 2016).

In addition, the *GPD1* and *GPD2* genes were disrupted to reduce glycerol as a by-product. The resulting strain (BD5_Ctnox_dGPD1dGPD2) produced 108.6 g/L of 2,3-butanediol from glucose with a yield (0.462 g 2,3-BD/g glucose) (Kim thesis., 2016).

Meanwhile, there is a research about production of R,R-2,3-BD in an industrial yeast. The *PDC1, PDC6, ADH1* gene of the *S. cerevisiae* were deleted to decrease ethanol production, and *alsS, alsD, BDH1* were overexpressed by the p413 yeast vector to produce R,R-2,3-BD effectively. Also, *noxE* gene was introduced to overcome the absence of ethanol production. As the result, the engineered strain produced 179 g/L of R,R-2,3-BD with the yield of 0.37 g/g and the productivity of 1.88 g/L/h. The highest productivity was 2.64 g/L/h during the fed batch fermentation. After deletion of *GPD1* and *GPD2* gene in this strain, the R,R-2,3-BD was produced with the titer of 152 g/L, the productivity of 0.95 g/L/h, and the yield of 0.41 g/g in the fed batch fermentation. (Lee thesis., 2018).

5. Research objectives

This study was focused on the production of meso-2,3-BD and the improvement of productivity. The specific objectives of this research are listed:

1) Production of meso-2,3-BD in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae* by introducing the meso-2,3-butanediol pathway.

2) Construction of a C_2 compound independent strain for improvement of productivity through expressing *CtPDC1*.

3) Improvement of meso-2,3-butanediol productivity by expressing *budC* additionally.

Ⅱ. Materials and Methods

1. Reagents

All chemicals were a reagent grade. Glucose, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), acetoin and meso-2,3-butanediol were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Bacto-peptone, bacto-trypton, yeast extract and bacto-agar were bought from Difco (Detroit, MD., USA); ethanol from Merck (Darmstadt, Germany); ampicillin from Fisher Scientific (Hampton, NH, USA); hygromycin B from Duchefa (Haarlem, Netherlands); aureobasidin A from Clontech Laboratories (Mountain view, CA, USA); ethidium bromide from Bioneer (Daejeon, Korea); agarose from Dongin Genomic (Seoul, Korea); NaOH, HCl, NaCl and H_2SO_4 from Duksan (Ansan, Korea).

2. Strains and plasmids

2.1. Strains

The Strains used in this study are listed in **Table 2**. *S. cerevisiae* D452-2 (*Matα, leu2, his3, ura3, can1*) (Hosaka *et al.* 1992) and the Pdc-deficient *S. cerevisiae* D452-2 strain (SOS5) were used as host strains for the genome editing and expression of the meso-2,3-butanediol biosynthesis pathway. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used for gene cloning and manipulation.

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

Table 1. List of strains used in this study.

Name	Description	Reference
D452-2	*S. cerevisiae* Matα leu2 his3 ura3 can1	(Hosaka *et al.* 1992)
SOS5	D452-2 *pdc1*Δ *pdc5*Δ *pdc6*Δ	(Kim *et al.* 2016)
S 5 dG SDN	SOS5, *gpd1*Δ *pgd2*Δ, p423TDH3_alsSalsD and p426TDH3_noxE	By JW Kim
S5 dG dB SDN	SOS5, *gpd1*Δ *pgd2*Δ *bdh1*Δ, p423TDH3_alsSalsD, p426TDH3_noxE	In this study
S5 dG dB SDNC	SOS5, *gpd1*Δ *pgd2*Δ *bdh1*Δ, p423TDH3_alsSalsD, p426TDH3_noxE p425TDH3_budC	In this study
S5 dG dB::G2Ct SDNC	SOS5, *gpd1*Δ *pgd2*Δ *dh1*Δ*::GPD2p_CtPDC1, P42*3TDH3_alsSalsD, p426TDH3_noxE p425TDH3_budC	In this study
S5 dG1::T3bC dG2 dB::G2Ct SDNC	SOS5, *gpd1*Δ*::TDH3p_budC gpd2*Δ *bdh1*Δ*::GPD2p_CtPDC1,* p423TDH3_alsSalsD, p426TDH3_noxE p425TDH3_budC	In this study

2.2. Plasmids

Yeast episomal plasmids (p423TDH3, p425TDH3, p426TDH3) harboring the *2 μ* origin, constitutive TDH3 promoter and CYC1 terminator from *S. cerevisiae* were used as mother vectors. To express the meso-2,3-butanediol biosynthesis pathway, *alsS* and *alsD* from *B. subtilis*, *budC* from *K. oxytoca, noxE* from *L. lactis,* are introduced to the mother vectors, respectively.

For disruption of genes involved in the competitive pathways using CRISPR-Cas9, a yeast centromere plasmid harboring the Cas9 expression cassette under the constitutive TEF1 promoter and CYC1 terminator from *S. cerevisiae* and yeast episomal plasmids harboring the guide-RNA expression cassette under the SNR52 promoter and SUP4 terminator are used. The plasmids and oligonucleotide sequences as the polymerase chain reaction (PCR) primer used in this study are described in Table 3 and 4, respectively.

Table 2. List of plasmids used in this study.

Name	Description	Reference
pRS426GPD	*URA3*, *GPD* promoter, *CYC1* terminator, 2μ origin, Amp^r	(Christianson *et al.* 1992)
pRS423GPD	*HIS3*, *GPD* promoter, *CYC1* terminator, 2μ origin, Amp^r	(Christianson *et al.* 1992)
pRS425GPD	*LEU2*, *GPD* promoter, *CYC1* terminator, 2μ origin, Amp^r	(Christianson *et al.* 1992)
pRS414AUR	*AUR1-C*, *GPD2* promoter, *CYC1* terminator, Amp^r	(Kim *thesis.* 2016)
p423_alsSalsD	pRS423GPD harboring *alsS* and *alsD* gene from *B. subtilis* str.168	(Kim *et al.* 2014)
p425_budC	pRS425GPD harboring *budC* gene from *K. oxytoca*	In this study
P426_noxE	pRS426GPD harboring *noxE* gene from *L. latis*	Construced by JW Kim
pAUR_Cas9	AUR1-C, CEN6, ARS4, Amp^R, P_{TEF1}_Cas9_T_{CYC1}	Consturcted by JW Kim
p42H_gBDH1	Hyg^R, 2 μ origin, Amp^R, P_{SNR32}_gBDH1_T_{SUP4}	Constructed by KM Lee
P41A_CtPCD1	pRS414AUR harboring *CtPDC1* gene form *Candida tropicalis*	Constructed by JW Kim
P42H_gGPD1	Hyg^R, 2 μ origin, Amp^R, P_{SNR32}_gGPD1_T_{SUP4}	In this study

Table 3. List of oligonucleotides used in this study.

Primer name	Oligonucleotide seuquence (5’ → 3’)
F_BamH1_budC	cgggatccatgaaaaaagtcgcactcgtgaccg
R_Hind3_budC	cccaagcttttagctaaataccatgccgccatcg
F1_Sac1_gRNA	ccgcgagctctctttgaaaagataatgtatg
R2_KpnI_gblock	tatagagcggccgcggtaccagacataaaaaacaaaaaaag
F2_BDH1_gRNA	attatcgacgtctcttggtgtgttttagagctagaaatagcaagt
R1_BDH1_gRNA	caccaagagacgtcgataatgatcatttatctttcactgcg
F3_BDH1_repair_GPD2p	tgatatccctaggccagaaatccaaaccgacgatgaggttattatcgacgca aaaacgacatatctattatagtggggag
R3_BDH1_repair_CYC1t	Accatccaagtactcgtgaagatccgagccacaaatcccacaccaagagag gccgcaaattaaagccttcg
R1_protospacer2_GPD1	tacaattttcggcaaccaccgatcatttatctttcactgcg
F2_protospacer2_GPD1	ggtggttgccgaaaattgtagttttagagctagaaatagcaagt
F3_GPD1_repair_TDH3	ctgtgattggatctggtaactggggtactactattgccaaggtggttgcctcatt atcaatactcgccatttcaaagaa
R3_GPD1_repair_CYC1	cacatttgtactattggagcgaaaacttctgggtatcccttacaattttcgcaaat taaagccttcgagcg

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 purchased from Takara (Tokyo, Japan).

3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) to amplify the gene for cloning was carried out with the PrimeSTAR® HS PCR PreMix (Takara, Shiga, Japan) in GeneAmp PCR System 2720 (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 carried out as follows; 1 cycle of 95°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 kb/min, 1 cycle of 72°C for 10 min. The amplified gene was carried out by gel electrophoresis.

Colony PCR to confirm the yeast transformation of plasmids was carried out with the TOPsimple™ DyeMIX-Tenuto PCR premix (enzynomics, Daejeon, Korea) in the same PCR machine. PCR

solution was composed of 10 pmol of forward and reverse primers and picked yeast colony as a template. PCR amplification was carried out as follows; 1 cycle of 98°C for 10 min; 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 kb/min, 1 cycle of 72°C for 10 min. The amplified gene was carried out through gel electrophoresis.

3.3. Preparation of plasmid DNA and bacteria genomic DNA

Mini-scale preparation of plasmid DNA was performed using *Dyne* ™ 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 performed as described by Sambrook (Sambrook et al. 1989). *E. coli* Top10 was cultured in 5 mL LB

medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) with 50 μg/ml of ampicillin 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 were spread on LB agar plates with an ampicillin selection marker.

3.5. Isolation of DNA fragments and DNA sequencing

DNA was digested with restriction enzymes and separated on a 0.1% (w/v) agarose gel. After full separation of the desired DNA band from the gel, the gel containing the DNA fragment was solubilized and further purified by using a Gel Extraction Kit from Takara (Tokyo, Japan). DNA sequencing was carried out by SolGent (Daejeon, Korea).

3.6. Yeast transformation

S. cerevisiae transformation was carried out through a standard lithium acetate transformation method (Gietz RD., Schiestl RH., 2007). A single colony of the *S. cerevisiae* was inoculated and incubated overnight in 5 mL YPD medium at 30°C. After 12 hour of growth, 2.5×108 cells of the cultures transferred to fresh 50 mL 2×YPD medium and cultured until $O.D._{600}$ reached 2. Cells harvested by centrifugation at 7000 rpm for 5 min at 4°C were washed twice with double-distilled water (DDW). Washed cells were mixed with 50% (w/v) PEG 3350, 1.0 M lithium acetate, carrier DNA, repair DNA and plasmid. They were conducted to heat-shock at 42°C for 45 min and incubated with 1 ml of YPD at 30°C for 2~4 hours. Transformants were selected on YNB medium containing 20 g/L glucose. Amino acids, nucleotides and antibiotics were added as necessary.

3.7. Genome editing by CRISPR-Cas9 system

Genome editing was conducted by CRISPR-Cas9. First, a plasmid (pAUR_Cas9) harboring the Cas9 protein from *Streptococcus pyogenes* was transformed to *S. cerevisiae*. Protospacers of target

genes were chosen using CRISPRdirect (http://crispr.dbcls.jp, Naito, Hino et al., 2015). For construction of a gRNA expressing plasmid, overlap-PCR was conducted. PCR primers including protospacer sequences (R1 and F2) were synthesized. The first fragment was amplified with F1 and R1 primer, and the other fragment was amplified with F2 and R2 primer. The full amplified fragment was obtained by the overlap-PCR using F1 and R2 primer. This fragment was cut by Sac Ⅰ and Kpn Ⅰ, and then ligated with the p42H plasmid.

Gene disruption was carried out through insertion a stop codon in the open reading frame. Repair DNA was designed to contain about 50 bp homology with the target gene and stop codon. Gene integration was performed by inserting a cassette of targeted gene with the sequence from promoter and terminator. Repair DNA was designed to contain about 50 bp homology with the forward and backward of target gene, respectively. Gene manipulation was carried out by introducing the gRNA expressing plasmid (p42H_gRNA) and a repair DNA. The conformation of the gene manipulation was confirmed through colony PCR and DNA sequencing (Zhang, Kong et al., 2014).

4. Media and culture conditions

4.1. Media

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

YP medium (10 g/L yeast extract and 20 g/L bacto-peptone) and YNB medium which lacked appropriate amino acids were used to select yeast strains. YNB Synthetic Complete medium (6.7 g/L yeast nitrogen base without amino acids and 2.0 g/L amino acids mixture without histidine, tryptophan, leucine or uracil) was used to cultivate yeast strains.

4.2. Batch fermentations in flask.

All cultures were performed at 30°C. Seed-cultures and pre-cultures of yeast cells were carried out aerobically in 5 mL test tubes and 250 mL baffled flasks respectively. Main flask batch cultures were performed in 250 mL flasks with a working volume of 50 mL at 80 rpm for oxygen-limited conditions. A solution of 0.5 g/.L ethanol was supplemented as a C_2-compound to support the growth of Pdc-deficient *S. cerevisiae* (Kim et al. 2015).

Seed cultures were prepared during for 48 hours through cultivation in YNB medium containing 20 g/L glucose with 0.5 g/L ethanol in a shaking incubator (Vision, Korea). The grown cells were transformed to 50 mL YNB medium containing 20 g/L glucose with 0.5 g/L ethanol. After cultivation, the mid-exponential phase cells were used as inoculums for the main culture. The inoculums cells were washed with double distilled water (ddH_2O) and transferred into the main cultures at the initial OD_{600} of 1.0. The main flask cultures were performed in YP medium (10 g/L yeast extract and 20 g/L bacto-peptone) containing 80 g/L glucose. Main cultures were performed in 250 mL flasks with a working volume of 50 mL at 80 rpm under oxygen-limited conditions.

4.3. Fermentations in bioreactor.

Seed cultures were prepared during for 48 hours by culturing in YNB medium containing 20 g/L glucose with 0.5 g/L ethanol using a shaking incubator (Vision, Korea). The grown cells were transformed to 200 mL YNB medium containing 20 g/L glucose with 0.5 g/L ethanol. After cultivation, the mid-exponential phase cells were used as inoculums for the main culture. The cells were harvested by

centrifugation at 7000 rpm for 5 min at 4℃ and washed in 5 mL of sterilized DDW and inoculated into a bioreactor with an initial OD_{600} of 10.0.

Fed-batch fermentations with bioreactor were carried out in 1500 ml YP medium containing 100 g/L glucose using 3 L-bench-top fermentor (Fermentec, Korea) and 500ml YP medium containing 100 g/L glucose using 1 L-bench-top fermentor (Fermentec, Korea) at 30℃. The medium pH was maintained at 5.5 with 5N NaOH and HCL solution and dissolved oxygen (DO) levels were monitored with O_2 sensor (Mettler Toledo, Switzerland). The culture medium was agitated at 300~500 rpm and aerated with air and NO_2 flow are of 1-2vvm with 1:1 ratio according to DO levels in medium. After depletion of glucose, additional sugar was added using 800 g/L glucose.

For the hydrolysate fermentation, 10 g/L yeast extract and 20 g/L peptone were added to the cassava hydrolysates from Changhae Ethanol Co., Ltd.

5. Analysis

5.1. Dry cell weight

Cell growth was estimated through gauging optical density (O.D.) at 600 nm using a spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea). Dry cell weight (DCW) was calculated by pre-estimated conversion equation.

$$\text{Dry cell weight (g/L)} = 0.3 \times \text{O.D.}_{600}$$

5.2. Metabolite detection

Concentrations of glucose, glycerol, acetate, meso-2,3-butanediol, acetoin and ethanol were measured through a high performance liquid chromatography (1260 Infinity, Agilent, Co, USA) equipped with a BioRad Aminex HPX-87H column (300 mm 7.8mm, 5 μm; Bio-Rad, Hercules, CA, USA). The column maintained at 30°C was eluted with 5 mM sulfuric acid at flow rate of 0.5 ml/min. Detection was made with a reflective index (RI) detector at 30°C.

5.3. Assay of stereoisomers of 2,3-BD using gas chromatograph

Stereoisomers of 2,3-BD were determined using a gas chromatography (GC) system (YL6100, YoungLin, Incheon, Korea) equipped with the HP-Chiral-20B GC column (30 m length, 0.32 mm inner diameter 0.25 μm film thickness, Agilent). Temperatures of an injector and a flame ionization detector were maintained at 225°C. Oven temperature was initially held at 40°C for 2min and increase at a rate of 5°C /min; isotherm at 45°C for 4 min; increase at a rate of 15°C /min; isotherm at 230°C for 4 min. The column was injected with 1 μL of the supernatant of culture broth in a splitless injection mode. Helium was used as the carrier gas at a 30 cm/sec constant flow. The FID was fed by a mixture of high purity air, hydrogen and helium. The injector and detector were maintained at 225°C. The column was injected with 1 μL of the supernatant of culture broth in a splitless injection mode. A mixture of (R/S)-acetoin, (R, R)-2,3-butanediol, and meso-2,3-butanediol (Sigma-aldrich) was prepared for a standard of stereoisomers.

5.4. *In vitro Pdc* activity analysis

To prepare crude extracts, about 1 x 10^9 mid-exponential phase cells grown on the YNB medium with 80 g/L glucose in a flask culture were harvested and washed twice with DDW. Protease inhibitor (Roche, Switzerland) was added and the harvested cells were lysed using Yeast Protein Extraction Reagent (Y-PER, Thermo Scientific, MA). After centrifugation for 20 min at 12,000 rpm and 4℃, the supernatants were used to measure the Pdc activity within 3 h and diluted with DDW if necessary. The NADH oxidase activity assays were performed at 30℃ with the reaction mixture containing 40mM imidazole hydrochloride buffer (pH 6.5), 5 mM $MgCl_2$, 0.2 mM TPP, 10U alcohol dehydrogenase from *S. cerevisiae*, 0.4 mM NADH, and 50 mM pyruvate (Postma et al., 1989). The reactions were initiated with addition pyruvate, and a decrease of absorbance at 340 nm was measured. One unit of activity was defined as the amount of enzyme oxidizing 1 μmol NADH per minute at the corresponding reaction conditions. The protein concentration of crude extracts was determined by the Bradford method (Bradford, 1976)

III. RESULTS AND DISCUSSIONS

1. Production of meso-2,3-BD in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*

1.1. Deletion of R,R-butanediol dehydrogenase by CRISPR Cas9

A wild type-yeast metabolizes most of glucose to produce ethanol and produces small amounts of R,R-2,3-butanediol. In previous studies, however, D452-2 was deleted with the ethanol metabolism pathway (SOS5). The acetolactate synthesize (*alsS)* and acetolactate decarboxylase (*alsD*) from *B.subtilis* which convert pyruvate into R-acetoin were overexpressed in this pyruvate decarboxylase deficient *S. cerevisiae*. In addition, as the NADH re-generation pathway through ethanol synthesis in the yeast was broken, the *noxE* from *L. lactis* was introduced to maintain the redox balance in the strain. To prevent accumulation of glycerol as a byproduct, *GPD1* and *GPD2* were disrupted. Finally, the S5 dG SDN strain was constructed and this strain produces R,R-2,3-BD using the endogenous *BDH1* (Kim,

thesis, 2016).

To produce meso-2,3-BD in S5 dG SDN, *BDH1* which converts acetoin into R,R-2,3-BD has to be eliminated. In this study, the *BDH1* was disrupted by introducing the stop codon (TAA) into the ORF of this gene through the CRISPR-Cas9 (Figure 1). Finally, the engineered strain constant was named as S5 dG dB SDN and the control strain was S5 dG SDN.

To verify the effect of *BDH1* disruption, the batch culture of the engineered and control strains was conducted, and the profiles are displayed in Figure 3 (A), (B). The control strain (S5 dG SDN) produced 27.5 g/L of R,R-2,3-BD with 0.40 g/g of R,R,-2,3-BD yield. The *BDH1* disrupted strain (S5 dG dB SDN) produced 14.6 g/L acetoin with 0.40 g/g of acetoin yield and there was no longer R,R-2,3-BD production. This result means that the *S. cerevisiae* doesn't produce R,R-2,3-BD anymore and this strain can be used as a mother strain to produce meso-2,3-BD after introduction the enzyme converting acetoin into meso-2,3-BD.

1.2. Introduction of meso-2,3-BD pathway by expressing *K. oxytoca budC* in Pdc-deficient S. cerevisiae

The competitive pathway was removed successfully, and then it was needed to introduce the enzyme converting R-acetoin into meso-2,3-BD into the S5 dG dB SDN. It was conducted to search for the gene expressing the enzyme and introduce it into S5 dG dB SDN.

The enzyme converting R-acetoin into meso-2,3-BD is called meso-2,3-butanediol dehydrogenase or acetoin reductase. Representative bacteria known as a native producer of meso-2,3-butanediol including *E. aerogene, K. pneumonia, R. oritihius, and K. oxytoca* have this enzyme coded for the *budC* gene (Table 4). Among these bacteria, *K. oxytoca* is knows as Risk Group 1 according to NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (April 2016). For this reason, the *budC* gene from *K. oxytoca* was selected, and it was over-expressed on the p425 vector using the TDH3 promoter which is a strong constitutive promoter. Finally, the engineered strain, S5 dG dB SDNC, was constructed.

When cultured in YPD medium under oxygen-limited conditions,

the control strain (S5 dG dB SDN) produced 14.6 g/L acetoin as a major metabolite, whereas the S5 dG dB SDNC strain able to overexpress the meso-2,3-BD biosynthetic genes produced 34.8 g/L meso-2,3-BD without production of R,R-2,3-BD.

In addition, the fermentation products were analyzed by GC to identify stereoisomers of 2,3-BD which were not separated by HPLC. As a result, it was confirmed that only meso-2,3-BD was produced without producing R,R-2,3-BD (Figure 6).

Finally, it was confirmed that the meso-2,3-BD production pathway was constructed in the yeast successfully.

Table 4. List of bacteria able to produce meso-2,3-BD

	Risk Group	Strain	Gene
meso-2,3-BD	RG 2	*K. pneumoniae*	*budC*
	RG 1	*K. oxytoca*	*budC*
	RG 2	*E. aerogenes*	*budC*
	RG 2	*R. ornithinolytica*	*budC*

Figure 1. Point mutation on *BDH1* genes by CRISPR-Cas9

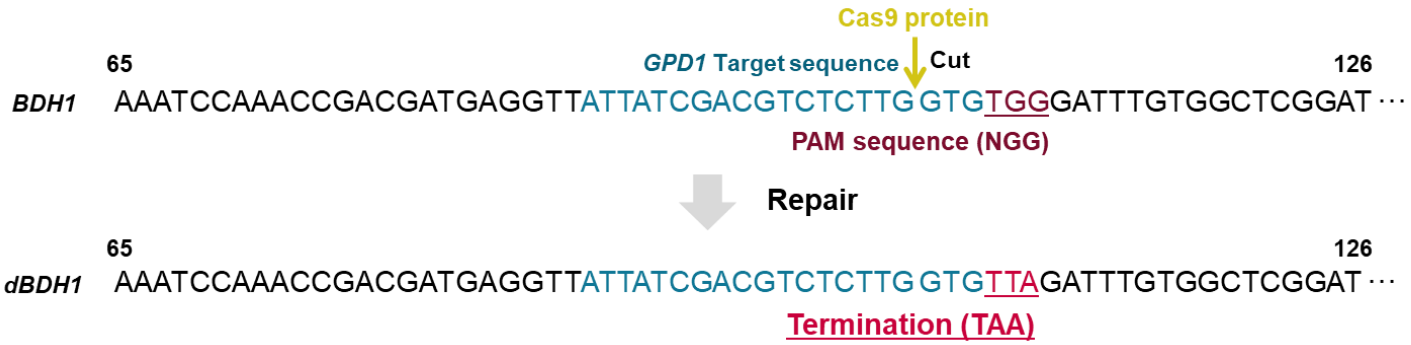
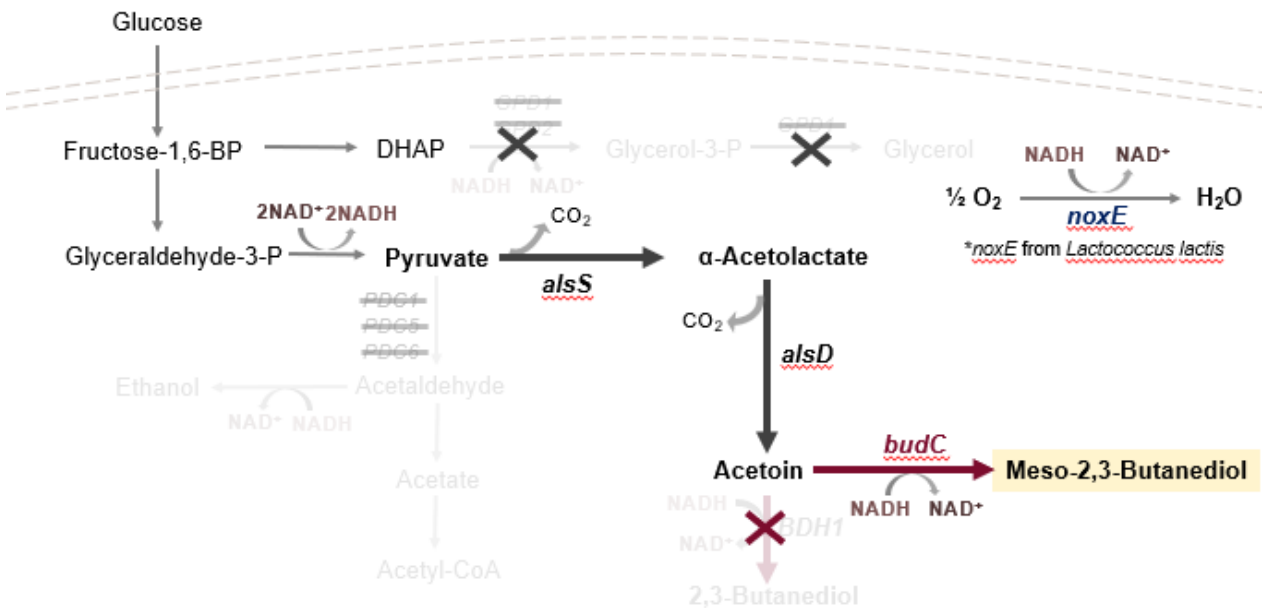


Figure 2. The pathway of 2,3-BD in *S. cerevisiae* and the strategies of construction of meso-2,3-BD synthetic pathway in *S. cesevisiae*.



1.3. Fed-batch fermentation of the engineered strains disrupted *BDH1* and expressing *budC*

To determine the industrial potential of the S5 dG dB SDNC strain as a meso-2,3-BD producer, fed-batch fermentations were carried out through intermittent addition of glucose under the optimum aeration condition (Table 6). Initial fermentation conditions were carried out at 400 rpm 2 vvm. When the rate of acetoin accumulation increased in the latter half of fermentation, the air condition is changed to 300 rpm 1 vvm. 2 g/L Ethanol was added with inoculation of the strain.

The final concentration of meso-2,3-BD was 133 g/L after 89 h cultivation, with a meso-2,3-BD yield (0.43 g meso-2,3-BD/g glucose) and volumetric productivity (1.49 g meso-2,3-BD/L·h). In addition, by changing the air condition in the latter half of fermentation, the production rate of acetoin slowed down and the accumulated acetoin was converted to meso-2,3-BD which resulted in the improvement of meso-2,3-BD productivity. (Figure 4)

1.4. Batch fermentation of the engineered strains using cassava hydrolysates

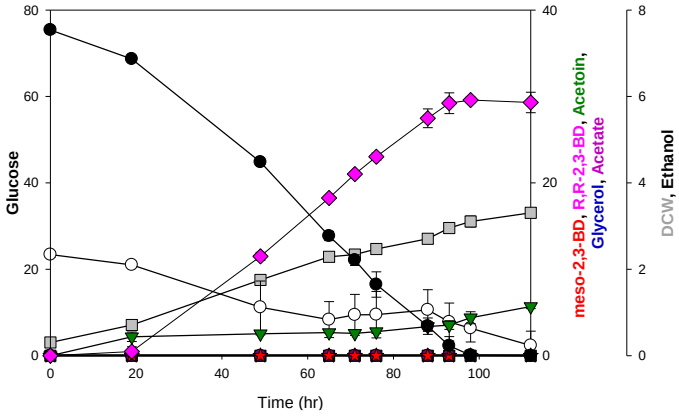
So far, the strain producing meso-2,3-BD, S5 dG dB SDNC, has been constructed and its ability of meso-2,3-BD production has been confirmed by batch cultures in a flask and fed-batch culture in a fermenter using purified glucose as a carbon source. However, when industrial fermentation is carried out, it is inefficient in terms of cost to use the purified glucose. As an alternative, pretreated-cassava hydrolysate could be used as a carbon source. In this part, the experiment was carried out to confirm that the engineered strain could be used industrially by using cassava hydrolysate as a sugar source instead of purified glucose.

In a 3L fermenter, 0.5 L of YP medium and 0.5 L of cassava hydrolysates were used. 2 g/L of ethanol was added as an initial medium condition and no further addition was made.

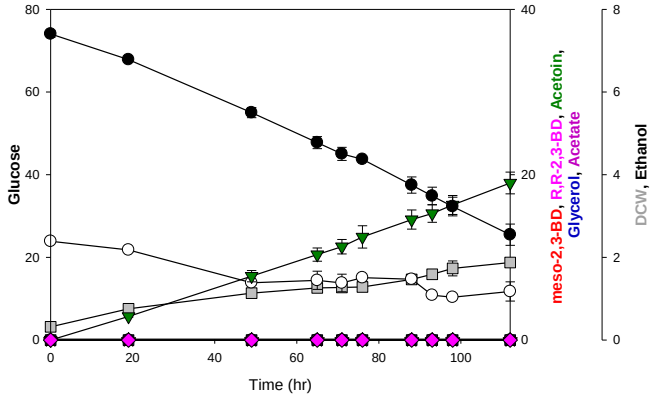
As a result, 146.6 g/L of sugar was consumed in 46 hours to produce 69.2 g/L of meso-2,3-BD representing a yield of 0.47 g/g and a productivity of 1.50 g/l/h. (Figure 5)

Finally, it was shown that the ability of meso-2,3-BD production of the

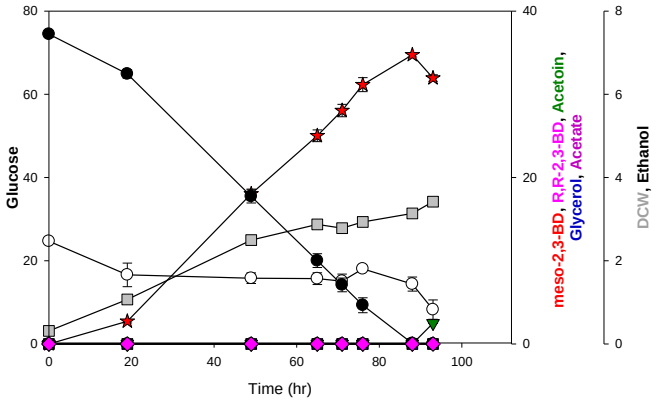
strain, S5 dG dB SDNC, was maintained when it used an unpurified sugar as a sole energy source and it means that this strain is of industrial utility value.


Glucose
meso-2,3-BD, R,R-2,3-BD, Acetoin,
Glycerol, Acetate
DCW, Ethanol
Time (hr)

(A)


Glucose
meso-2,3-BD, R,R-2,3-BD, Acetoin,
Glycerol, Acetate
DCW, Ethanol
Time (hr)

(B)


Glucose
meso-2,3-BD, R,R-2,3-BD, Acetoin,
Glycerol, Acetate
DCW, Ethanol
Time (hr)

(C)

Figure 3. Batch fermentation profiles of (A) S5 dG SDN, (B) S5 dG dB SDN, and (C) S5 dG dB SDNC strains cultured in flask.

Symbols : Glucose (), DCW (), Glycerol (), Acetate (), R,R-2,3-Butanediol (), meso-2,3-Butanediol (), Acetoin (), Ethanol ()

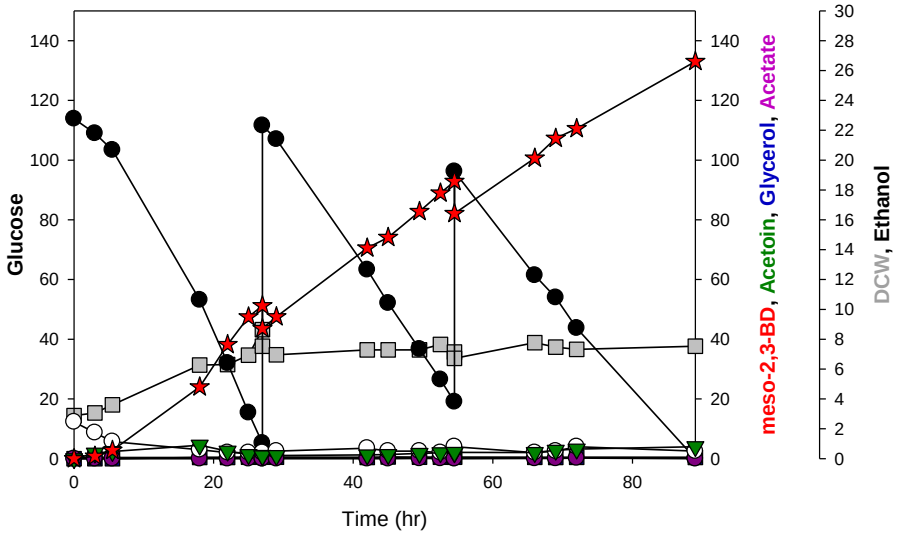


Figure 4. Fed batch fermentation profiles of S5 dG dB SDNC strain cultured in fermenter with glucose.

Symbols : Glucose (●), DCW (□), Glycerol (■), Acetate (●), meso-2,3-Butanediol (★), Acetoin (▼)Ethanol (○)

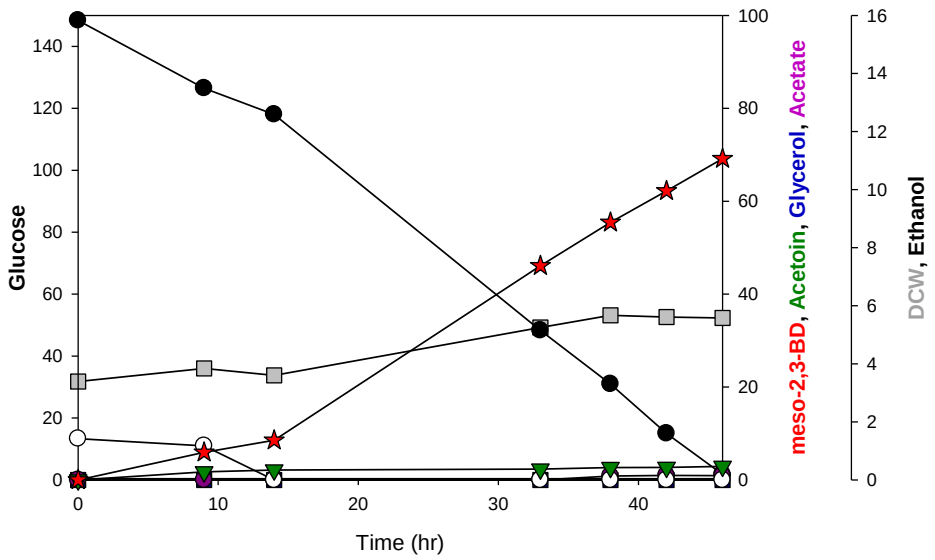


Figure 5. Batch fermentation profiles of the S5 dG dB SDNC strain cultured in fermenter with cassava hydrolysates.

Symbols : Glucose (●), DCW (□), Glycerol (■), Acetate (●), meso-2,3-Butanediol (★), Acetoin (▼)Ethanol (○)

Table 5. Summary of flask cultivation in part 1.1 and 1.2.

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)			Yield (g product/g glucose)			Productivity (g/L/hr)		
				meso-2,3-BD	R,R-2,3-BD	Acetoin	meso-2,3-BD	R,R-2,3-BD	Acetoin	meso-2,3-BD	R,R-2,3-BD	Acetoin
A	93	69	2.7	0	27.5	3.38	0	0.40	0.05	0	0.31	0.04
B		37	1.5	0	0	14.6	0	0	0.40	0	0	0.17
C		74	3.2	34.8	0	0	0.47	0	0	0.40	0	0

Table 6. Summary of fed batch cultivation in part 1.3.

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)			Yield (g product/g glucose)		Productivity (g/L/hr)	
				meso-2,3-BD	Acetoin	Ethanol	meso-2,3-BD	Acetoin	meso-2,3-BD	Acetoin
S5 dG dB SDNC	54	212	7.1	92.9	2.0	0	0.43	0.01	1.70	0.03
	89	308	7.5	133	4.0	0	0.43	0.01	1.49	0.04

Table 7. Summary of batch cultivation in part 1.4.

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)			Yield (g product/g glucose)		Productivity (g/L/hr)	
				meso-2,3-BD	Acetoin	Ethanol	meso-2,3-BD	Acetoin	meso-2,3-BD	Acetoin
S5 dG dB SDNC	46	147	5.6	69.2	2.9	0	0.47	0.02	1.50	0.06

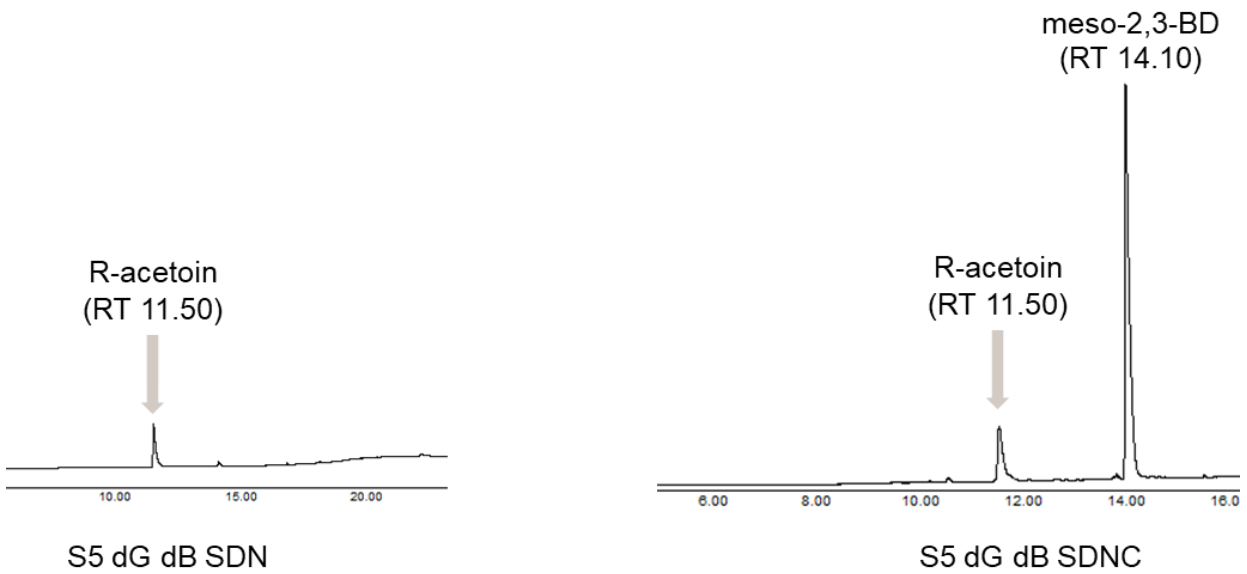


Figure 6. Confirmation of stereotypes of 2,3-BD by GC in part 1.2.

2. Construction of C_2 compound independent strain

2.1. Integration of *CtPDC1* by CRISPR Cas9 system

The potential defects of PDC deficient *S. cerevisiae* include a need for external supplement of two-carbon compounds such as ethanol for acetyl-CoA synthesis which is used to synthesize fatty acids and lysine (Flikweert et al, 1996). In the previous study, the expression of *C. tropicalis PDC1 (CtPDC1)* was conducted with diverse combination of promoters and yeast vectors. When the *CtPCD1* was expressed by a p406GPD2 vector, the *S. cerevisiae* produced the high ratio of 2,3-BD without additional ethanol production. As a result, two-carbon independent cell growth was observed (Kim thesis, 2016).

In this study, therefore, to produce 2,3-BD without a two-carbon supplement such as ethanol, Ct*PDC1* was expressed by integration using a GPD2 promoter. It is conducted by CRISPR Cas9 and the 'GPD2 p-CtPPDC1-CYC1t' cassette was integrated into the ORF of the *BDH1* of the S5 dG dB SDNC strain. The constructed strain is called S5 dG dB::G2Ct SDNC.

Flask batch cultivations were carried in YP medium with 80 g/L glucose without ethanol under micro-aerobic conditions. The results of batch fermentation were shown in Figure 8. With expression of *CtPDC1*, the biomass yield of 2.64 g DCW / g glucose and the titer of 24.0 g/L were obtained during 112 h cultivation, while the control was 1.52 g DCW/g and 10.5 g/L. The productivity of meso-2,3-BD was respectively 0.21 g/L/h and 0.09 g/L/h.

Additionally, *in vitro pdc* assay was conducted to confirm the effect of *CtPDC1*. As shown in Figure 9, NADH oxidase activity of S5 dG dB::G2Ct SDNC strain was measured 164.4 U/mg-protein, on the other hand, S5 dG dB SDNC strain as a control was measured 4.51 U/mg-protein. Although the *PDC1, PDC5*, and *PDC6* were deleted, the activity was measured with a low level. It means that there are activities of PDC isozymes that can convert NADH into NAD^+ using pyruvate.

In this study, the engineered strain represented the improvement of growth and the production of meso-2,3-BD without ethanol supplement, whereas the control strain stopped to grow and produce meso-2,3-BD in the middle of the fermentation. As a result, a two-carbon independent strain was constructed successfully.

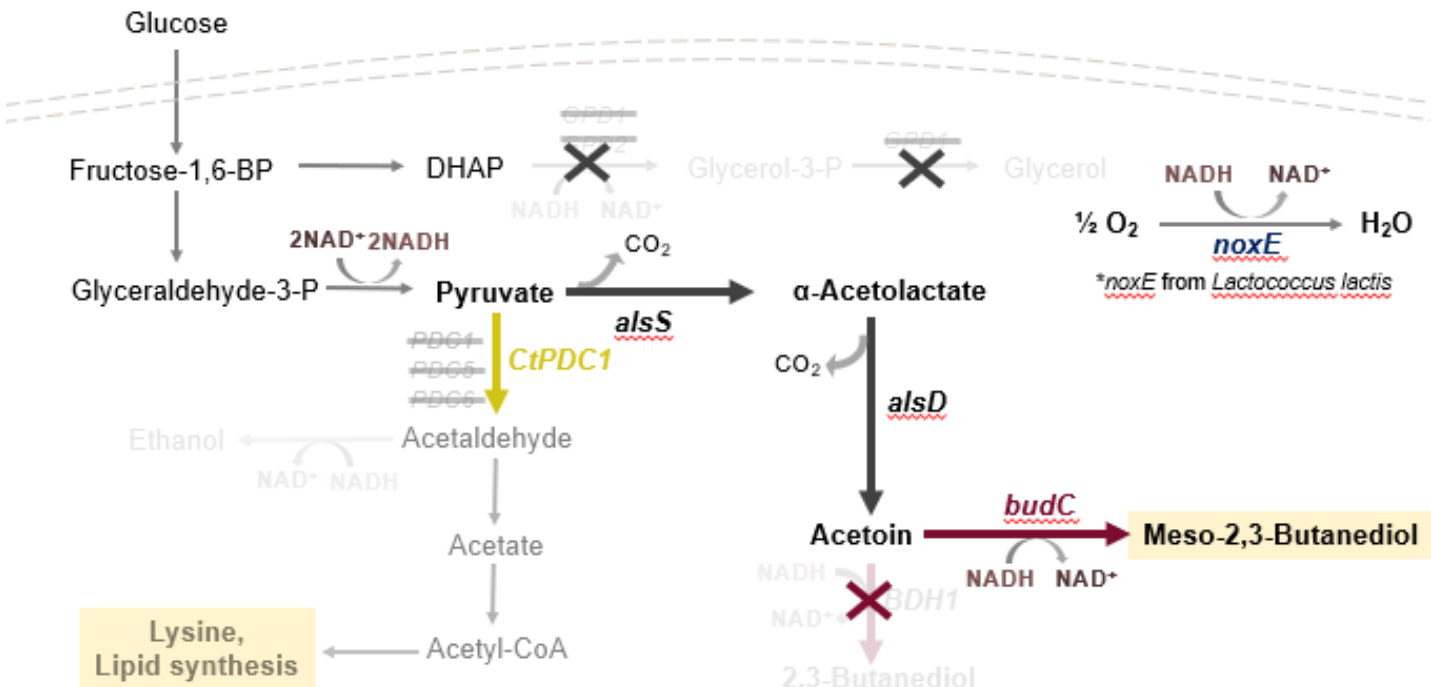
2.2. Fed-batch fermentation of the engineered strain integrated *CtPDC1*

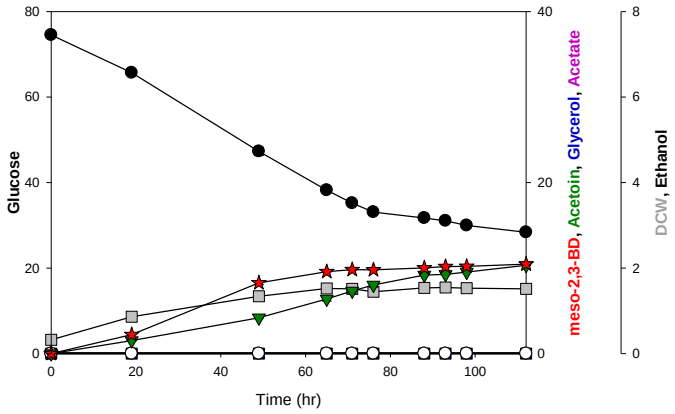
To evaluate the fermentation properties of the S5 dG dB::G2Ct SDNC strain, fed-batch fermentation was carried out without ethanol addition. The initial fermentation medium contained 100 g/L of glucose. In consideration of the characteristics of the NADH oxidase encoded by the *noxE* gene, agitation was changed during the cultivation: from 500 rpm to 300 rpm when the rate of acetoin accumulation increased. And the aeration condition was also changed from 2vvm to 1vvm with the ratio of N_2 : air = 1 : 1.

The fed-batch fermentation with the strain resulted in 131 g/L of meso-2,3-BD during 77 hours of cultivation with a yield of 0.45 g meso-2,3-BD/g glucose and a productivity of 1.67 g/L/h. In addition, by changing the air condition in the latter half of fermentation, the production rate of acetoin was slow down and the accumulated acetoin was converted to meso-2,3-BD. (Figure 10)

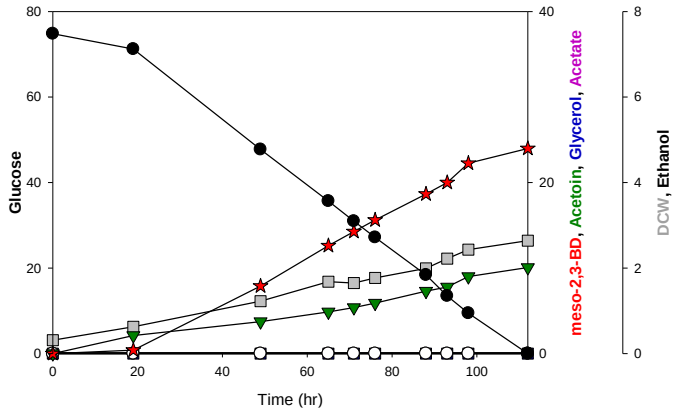
This result indicated that the growth of the strain and the productivity of meso-2,3-BD did not deteriorate even when the ethanol was not supplied.

Figure 7. The strategies of construction of C_2 compound independent strain.





(A)



(B)

Figure 8. Batch fermentation profiles of the (A) S5 dG dB SDNC and (B) S5 dG dB::G2Ct SDNC strains cultured in flask.

Symbols : Glucose (●), DCW (□), Glycerol (■), Acetate (●), meso-2,3-Butanediol (★), Acetoin (▼)Ethanol (○)

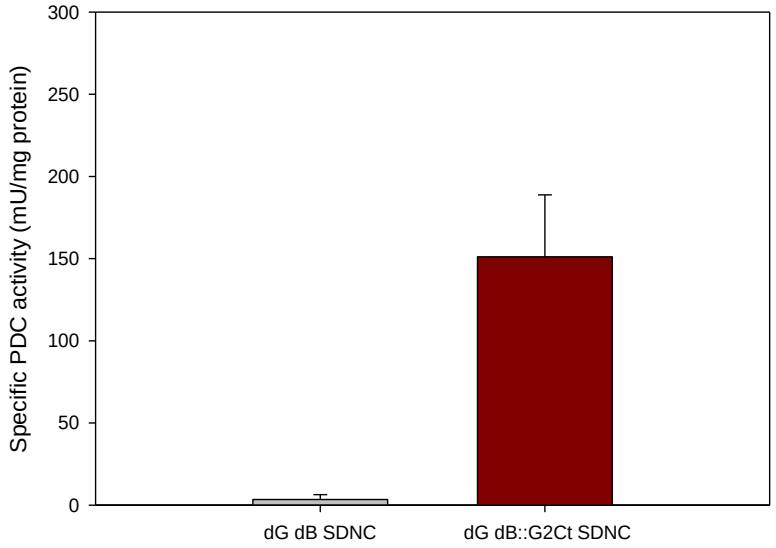


Figure 9. *In vitro pdc* activity assay for S5 dG dB SDNC strain and S5 dG dB::G2Ct SDNC strain.

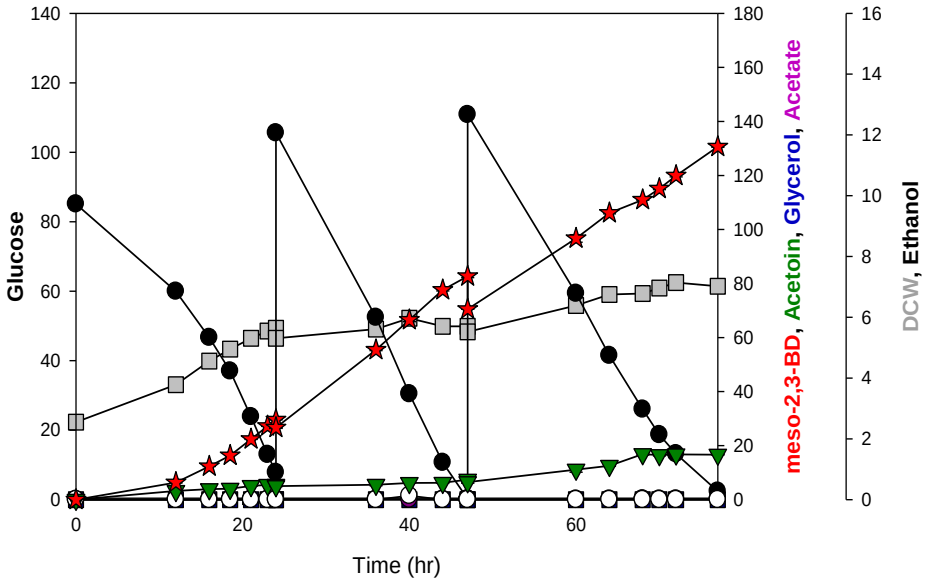


Figure 10. Fed batch fermentation profiles of the S5 dG dB::G2Ct SDNC strain cultured in fermenter.

Symbols : Glucose (●), DCW (□), Glycerol (■), Acetate (●), meso-2,3-Butanediol (★), Acetoin (▼)Ethanol (○)

Table 8. Summary of flask cultivation in part 2.1

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)			Yield (g product/g glucose)		Productivity (g/L/hr)	
				meso-2,3-BD	Acetoin	Ethanol	meso-2,3-BD	Acetoin	meso-2,3-BD	Acetoin
A	112	46.1	1.52	10.5	10.3	0	0.23	0.22	0.09	0.09
B		74.8	2.64	24.0	10.1	0	0.31	0.14	0.21	0.09

Table 9. Summary of fed batch cultivation in part 2.2

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)			Yield (g product/g glucose)		Productivity (g/L/hr)	
				meso-2,3-BD	Acetoin	Ethanol	meso-2,3-BD	Acetoin	meso-2,3-BD	Acetoin
S5 dGdB::G2CtSDNC	77	291	7.02	131	16.6	0	0.45	0.06	1.67	0.22

3. Improvement of meso-2,3-butanediol productivity by expressing *budC* additionally

3.1. Integration of *budC* by CRISPR Cas9 system

For the Pdc-deficient *S. cerevisiae*, the NADH oxidase (*noxE*) was further expressed to maintain the NAD^+/NADH balance. This NADH oxidase uses oxygen as a substrate to re-oxidize NADH instead of PDC to maintain its reducing power in a yeast. However, as the growth of microorganisms enters the stationary phase and death phase, the amount of oxygen required for growth is lowered, leading to an increase in the substrate of *noxE* and excessive oxidation of NADH. However, because the acetoin reductase (*budC*), which produces meso-2,3-BD, also uses NADH as a substrate, the higher the activity of *noxE*, the less NADH used by *budC* and resulting in acetoin accumulation.

Therefore, in order to overcome this limitation, experiments were carried out to increase the activity of acetoin reductase (*budC*) by further expressing the enzyme that converts acetoin to meso-2,3-BD for reducing the accumulation of by-products and increasing productivity.

In addition, another reason for accumulation of acetoin is the stability of p425, a vector expressing the *budC* gene, because the vector is known

as an unstable yeast vector. When the second subculture was performed on the YNB selective medium, it was confirmed that 25% of the vectors were lost. Therefore, integration into the chromosome can further express the *budC* gene and solve the instability problem of the plasmid.

For this purpose, a strong constitutive promoter, TDH3 promoter, was used and the cassette of 'TDH3p-budC-CYC1' integrated into the *GPD1* site through the CRISPR Cas9 system. The cassette was amplified by PCR using forward and reverse primers (F_TDH3_budC, R_CYC1_budC), and p425TDH3_budC as the template. The engineered strain, S5 dG1::T3bC dB::G2Ct SDNC, was confirmed by colony PCR using forward and reverse primers (F_check_GPD1, R_check_GPD1).

When cultured in YPD medium without ethanol under oxygen-limited conditions, the control strain (S5 dG dB::G2Ct SDNC) produced 20 g/L of meso-2,3-BD with 0.33 g/g of yield and 0.22 g/L/h of productivity, whereas the S5 dG1::T3bC dB::G2Ct SDNC strain produced 31.5 g/L meso-2,3-BD with 0.44 g/g of yield and 0.31 g/L/h of productivity, and the accumulation of acetoin also decreased (Figure 12).

These results suggest that additional expression and integration of *budC* is effective in reducing accumulation of acetoin and improving

yield and productivity for meso-2,3-BD production.

3.2. Fed-batch fermentation of the engineered strain integrated *budC*

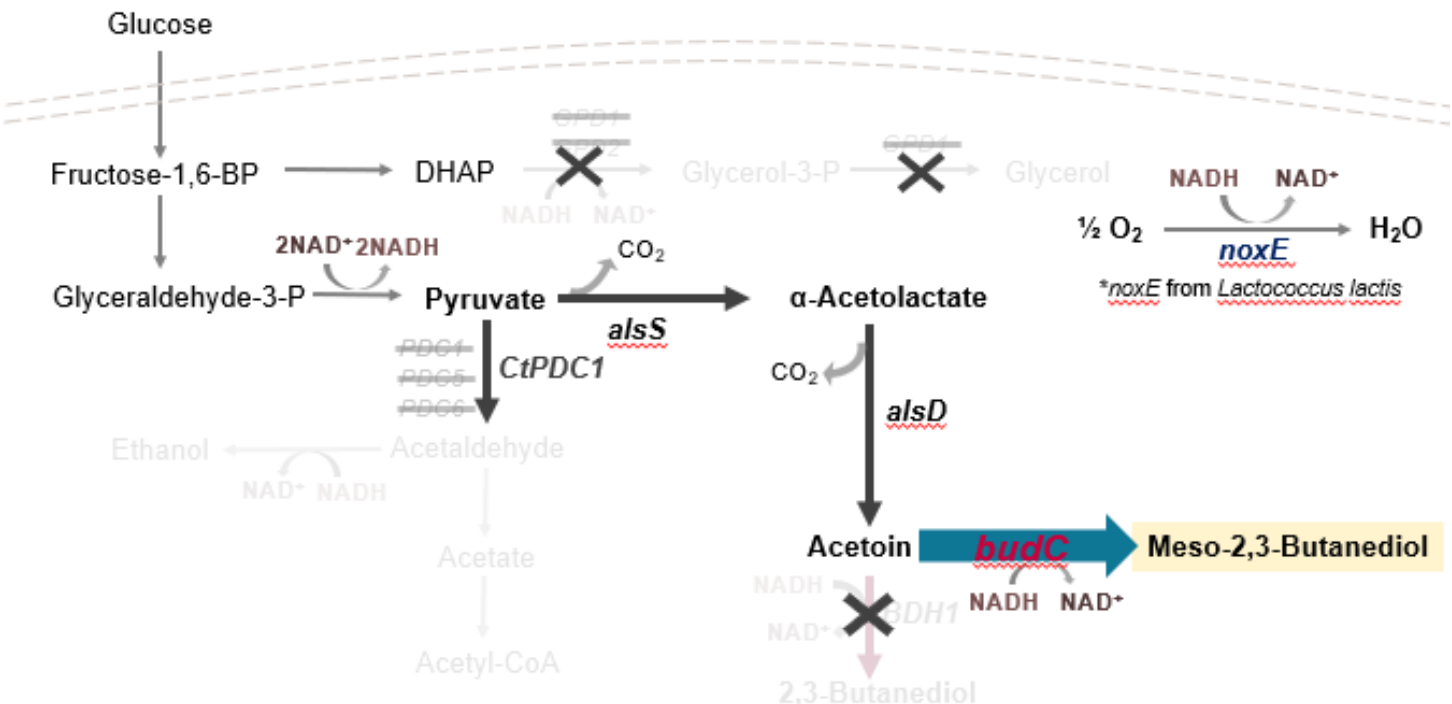
To evaluate the fermentation of the S5 dG::T3bC dB::G2Ct SDNC strain, fed-batch fermentation was carried out without ethanol. The initial fermentation medium contained 100 g/L of glucose. In consideration of the characteristics of the NADH oxidase encoded by the *noxE* gene, agitation was changed during the cultivation: from 500 rpm to 300 rpm when the rate of acetoin accumulation increased. And the aeration condition was also changed from 2vvm to 1vvm with the ratio of N_2 : air = 1 : 1.

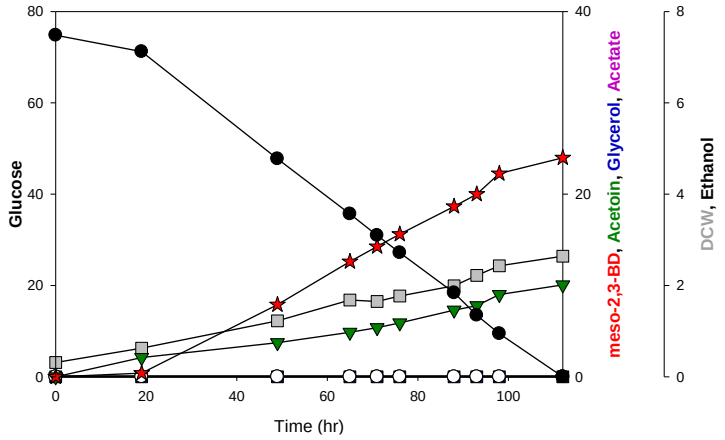
The fed-batch fermentation with the strain resulted in 171 g/L of meso-2,3-BD during 95 hours of cultivation with a yield of 0.49 g meso-2,3-BD/ g glucose and a productivity of 1.80 g/L/h. In addition, by changing the air condition in the latter half of fermentation, the production rate of acetoin slowed down and the accumulated acetoin was converted to meso-2,3-BD.

As a result, the yield of 0.49 g/g corresponds to 98% of the theoretical yield of 0.5 g/g and this means that the consumed glucose was mostly used in the production of meso-2,3-BD in addition to the

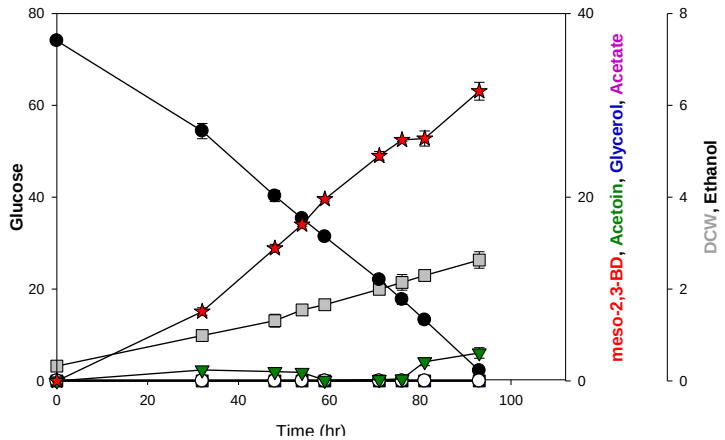
growth of bacteria, thereby minimizing accumulation of by-products and producing meso-2,3-BD with high purity (Figure 13).

Figure 11. Strategies for improving yield and productivity by expression *budC* additionally.





(A)



(B)

Figure 12. Batch fermentation profiles of the (A) S5 dG dB::G2Ct strain and (B) S5 dG::T3bC dB::G2Ct SDNC strains cultured in flask.

Symbols : Glucose (●), DCW (□), Glycerol (■), Acetate (●), meso-2,3-Butanediol (★), Acetoin (▼)Ethanol (○)

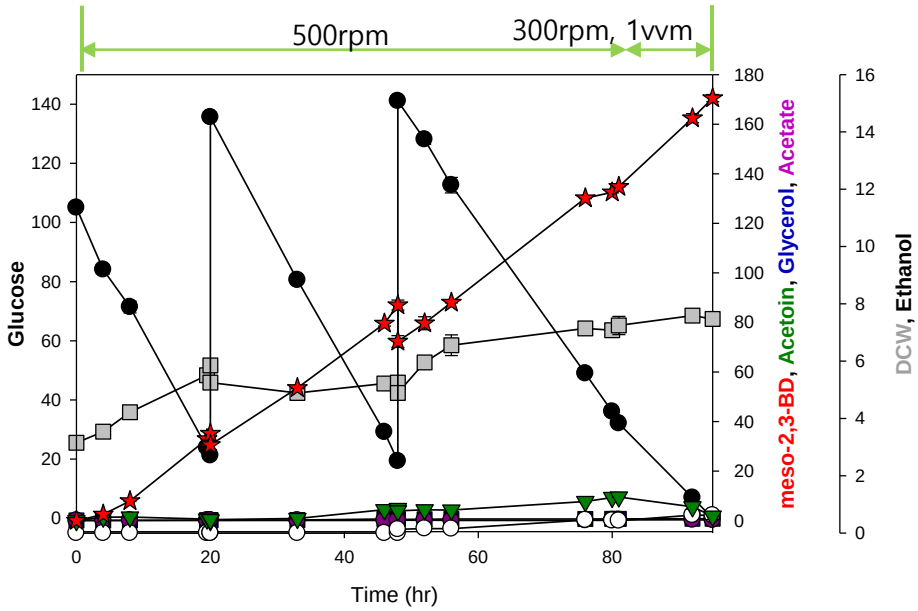


Figure 13. Fed batch fermentation profiles of S5 dG::T3bC dB::G2Ct SDNC strain cultured in fermenter.

Symbols : Glucose (●), DCW (□), Glycerol (■), Acetate (⬢), meso-2,3-Butanediol (★), Acetoin (▼)Ethanol (○)

Table 10. Summary of flask cultivation in part 3.1.

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)			Yield (g product/g glucose)		Productivity (g/L/hr)	
				meso-2,3-BD	Acetoin	Ethanol	meso-2,3-BD	Acetoin	meso-2,3-BD	Acetoin
A	93	61.4	2.22	20.0	7.78	0	0.33	0.13	0.22	0.08
B		71.8	2.63	31.5	3.05	0	0.44	0.04	0.34	0.03

Table 11. Summary of fed batch cultivation in part 3.2

Strain	Hour	Total consumed glucose (g/L)	Dry cell weight (g/L)	Titer (g/L)		Yield (g product/g glucose)			Productivity (g/L/hr)	
				meso-2,3-BD	Acetoin	Ethanol	meso-2,3-BD	Acetoin	meso-2,3-BD	Acetoin
S5 dG::T3bC dB::G2Ct SDNC	95	341	7.47	171	1.80	0.62	0.49	0.01	1.80	0.02

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) To eliminate the R,R-2,3-BD synthetic pathway, the endogenous *BDH1* gene was disrupted by CRISPR-Cas9. To produce meso-2,3-BD, the *budC* gene from *K. oxytoca* was re-introduced. The constructed *S. cerevisiae* S5 dG dB SDNC produced 133 g/L of meso-2,3-BD with 0.43 g/g of yield.

(2) To solve the C_2-dependent growth of 2,3-BD-producing Pdc-deficient *S. cerevisiae* on glucose media, the *PDC1 g*ene from *C. tropicalis* was expressed in the S5 dG dB SDNC strain. The resulting S5 dG dB::G2Ct SDNC strain produced 131 g/L of meso-2,3-BD from glucose with 1.70 g/L/h of productivity in a fed-batch fermentation without addition of ethanol.

(3) To improve a yield and a productivity, the *budC* gene was integrated to the chromosome of the S5 dG dB::G2Ct SDNC strain. The engineered strain, S5 dG::T3bC dB::G2Ct SDNC, produced 171 g/L of meso-2,3-BD with a yield of 0.49 g/g corresponding to 98% of the theoretical yield.

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국 문 초 록

지구 온도가 상승하는 지구온난화는 전 지구적 차원의 환경문제이다. 하지만, 화석연료의 사용으로 인한 이산화탄소 배출량은 점점 증가하고 있다.

이에따라, 화학연료 기반의 시스템을 대체할 바이오매스 기반의 시스템이 주목받고 있다. 바이오 매스는 전처리를 통하여 단당류로 전환되고 이것을 미생물이 사용함으로써 유용한 바이오 연료 및 화학물질을 생산할 수 있다. 본 연구에서는 바이오매스 기반의 미생물공장화 기술을 구축하여 바이오 화학 소재를 생산하고자 하였다.

2,3-Butanediol (2,3-BD)는 식품 첨가제와 플라스틱 물질의 전구체로서 다양한 적용범위를 가지는 범용성 물질이다. 그 중 meso-2,3-BD는 항균성이 높고 보존력이 있어 화장품 첨가제나 작물 보호제로서 이용가치가 있다.

현재까지 미생물을 기반의 meso-2,3-BD를 생산은 주로 박테리아 기반의 생산에 관한 연구이다. 이것은 고수율로

2,3-BD를 생산할 수 있지만 이들 대부분이 병원성 균으로 분류되기 때문에 안전성의 문제가 제기 되며, 박테리오파지 등의 잡균에 감염이 쉬워 산업적인 공정을 구축하는데 어려움이 따른다. 그 대안으로 *Saccharomyces cerevisiae*는 GRAS (Generally Recognized As Safety) 미생물로서 안전하다고 알려져 있고, 상업적인 에탄올 생산에 이용되어 산업적 이용 가능성이 입증되었다. 하지만 야생형의 *S. cerevisiae*는 소량의 R,R-2,3-BD를 한다. 선행연구자에 의해 pyruvate decarboxylase 활성이 완전히 저해된 효모 (SOS5)에서 R,R-2,3-BD의 생산성을 높인 연구가 진행된 바 있다.

본 연구에서는 선행연구를 기반으로 하여, 에탄올 생합성 경로가 제거된 효모에서 meso-2,3-BD를 생산하는 연구를 수행하였다. 이를 위하여 효모에 meso-2,3-BD의 생산경로를 도입하고, meso-2,3-BD의 생산에 있어 높은 수율과 생산성을 얻는 것이 최종 목표이다.

구체적으로는 선행연구자에 의해 구축된 pyruvate

decarboxylase 활성이 저해된 효모(SOS5)에 2,3-BD 생합성 경로를 도입한 균주 (SOS5 dG SDN)를 모 균주로 선택하였다. 이 균주는 도입된 *alsS*와 *alsD* 유전자에 의해 생성된 acetoin을 효모가 자체적으로 가지고 있는 *BDH1* 유전자를 이용하여 R,R-2,3-BD로 전환하는데, 이 유전자를 CRISPR Cas9 방법으로 파쇄 함으로써 R,R-2,3-BD가 생성되는 것을 방지하였다. 이후, acetoin을 meso-2,3-BD로 전환하는 유전자를 박테리아로부터 도입하였다. meso-2,3-BD를 생성한다고 알려져 있는 여러 박테리아 중 Risk group 1으로 알려진 *Klebsiella oxytoca*을 선택하였고, 이것의 *budC* 유전자를 플라스미드로 과발현하였다. 이렇게 구축된 균주를 플라스크에서 발효하여 그 효과를 확인하였다. 분리되지 않는 2,3-BD의 이성질체를 가스크로마토그래피 (GC)으로 정성 분석하여 R,R-2,3-BD는 생성하지 않으면서 meso-2,3-BD만 생성하는 것을 확인하였다.

다음으로, meso-2,3-BD의 합성의 수율과 생산성을 높이

기 위한 연구를 진행하였다. 첫째로, 에탄올 생합성 대사 과정이 모두 제거된 효모는 acetyl CoA, 아미노산 등 생장에 필수적인 물질을 합성하지 못한다. 이를 위해 발효 중 C_2 compound로써 에탄올 공급이 필요하다. 따라서, 에탄올이 없는 배지에서도 잘 자랄 수 있도록 C_2 compound에 비 의존적인 균주를 제작할 필요가 있다. 이를 위해 낮은 *PDC* 활성도를 가지는 *Candida tropicalis* 유래의 *PDC1*을 CRISPR Cas9 방법으로 *BDH1* 자리에 삽입하였다. 그 결과 회분식 발효에서 에탄올 공급 없이, 112 시간에 24.0 g/L의 meso-2,3-BD를 생산하였으며 대조군 대비 생산성이 2.3 배 향상되었다. *PDC* activity assay를 통해 *CtPDC1* 도입 전에 비해 PDC의 활성도가 증가한 것을 확인하였다. 이 후 대량생산을 위하여 발효조에서 유가식 발효를 진행 하였다. 그 결과 77시간에 131 g/L의 meso-2,3-BD를 생산하였고, 0.46 g/g의 수율과 1.71 g/L·h의 생산성을 나타내었다. 두번째로, 이전 발효 양상을 보면 발효 후반부로 갈수록 부산물로 acetoin이 축적되는

문제가 있어 이를 개선하는 연구를 하였다. 이러한 한계를 극복하기 위하여 acetoin을 meso-2,3-BD를 전환하는 *budC*를 추가로 발현하였다. 이를 위해, 강한 프로모터를 사용하였으며 CRISPR Cas9 system으로 염색체에 삽입하였다. 플라스크 수준으로 회분식 배양을 진행한 결과 대조군은 93시간에 20.0 g/L를 생산한 것에 비해 실험군은 31.5 g/L의 meso-2,3-BD를 생산하였고, 생산성이 1.6배 증가한 결과를 얻었다. 이 후 대량생산을 위해 발효조에서 유가식 배양을 진행하였다. 그 결과, 95시간에 171 g/L의 meso-2,3-BD를 생산하였다. 또한, 이론 수율의 98%에 해당하는 .49 g/g의 수율과 1.80 g/L/g의 생산성을 얻었다. 이를 통하여 재조합 *S. cerevisiae*인 S5 dG::T3bC dB::G2Ct SDNC 균주는 meso-2,3-BD를 고순도로 생산할 수 있는 균주임을 증명하였다.

본 연구를 통하여, 박테리아만 생산가능 했던 meso-2,3-BD를 효모에서 생산하였으며, 에탄올 생합성 경로가 삭제된 효모의 한계를 극복하고 부산물을 최소화함으로써 산

업적인 이용가치를 향상시켰다.

주요어 : 2,3-Butanediol, pyruvate decarboxylase, meso-2,3-butanediol, acetoin reductase, *Saccharomyces cerevisiae*

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