A Thesis for the Degree of Master of Science

Production of 2,3-butanediol from glucose and galactose in engineered *Saccharomyces cerevisiae*

재조합 효모를 이용한 포도당과 갈락토오스로부터
2,3-butanediol 생산

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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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工 學 碩 士 學 位 論 文

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指導敎授 徐 鎭 浩

이 論文을 工學碩士學位論文으로 提出함
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崔 銀 志

崔 銀 志 의 工學碩士 學位論文을 認准함

2016年 2月

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副委員長	서 진 호	(인)
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ABSTRACT

2,3-Butanediol (2,3-BD) is a platform chemical with extensive industrial applications since it can be converted into other valuable chemicals by dehydration, dehydrogenation, ketalization and esterification. Especially, 1,3-butadiene, a dehydration product of 2,3-BD, is a main substance used for producing synthetic rubber. Most microbial fermentations for 2,3-BD production have been focused on pathogenic bacteria, which makes large-scale fermentations difficult in terms of safety and industrialization. As an alternative, 2,3-BD production by a GRAS (Generally Regarded As Safe) microorganism *Saccharomyces cerevisiae* would be suitable.

Also, production of 2,3-BD from marine biomass is one of the key issues for economic viability of bio-based chemicals. Among various sugars, glucose and galactose are of special interest because they are abundant in marine biomass. One of the most important concerns in

mixed sugar fermentations is the catabolite repression that represses the enzyme expression to utilize sugars other than glucose.

Pyruvate decarboxylase(Pdc)-deficient *S. cerevisiae* (SOS2) was constructed to eliminate ethanol production. Then, the evolved SOS2 strain (SOS4) was obtained by serial cultivation in excess glucose medium for cell growth on glucose medium. Among the chromosomal mutations of the SOS4 strain, a single nucleotide polymorphism was found on the *MTH1* gene which functions as a glucose signal regulator.

Efficient 2,3-BD production using engineered strains capable of effective fermentation from glucose and galactose is the objective in this study.

First, the 2,3-BD-producing Pdc-deficient BD4 strain with the *MTH1* mutation was tested for a performance of 2,3-BD production in a batch fermentation, resulting in simultaneous utilization of glucose and galactose. Therefore, in order to evaluate if the *MTH1* mutation affects

the fermentation performances in a mixture of glucose and galactose, the evolved pdc-deficient strain (SOS2) and engineered strain by a point mutation on the *MTH1* gene (SOS2_Mth1) were used. The SOS2 and SOS2_Mth1 strains containing the *alsS* gene encoding α-acetolactate synthase and the *alsD* gene encoding α-acetolactate decarboxylase both from *Bacillus subtilis* and overexpression of the endogenous *BDH1* gene coding for 2,3-BD dehydrogenase were constructed (BD2 and BD2M) and tested for a performance of 2,3-BD production in batch fermentation under oxygen-limited conditions. The BD2M strain which is the *MTH1* mutant was able to co-ferment both sugars, resulting in increased 2,3-BD productivity by 41% compared to the control strain, BD2 strain. To evaluate the fermentation aspects of the BD2M strain in a bioreactor, fed-batch fermentation was carried out to obtain 75.5 g/L of 2,3-BD by simultaneous consumption of glucose and galactose. However, in the rest of the fermentation, galactose

consumption rate was substantially reduced compared with glucose consumption rate.

Second, to increase galactose uptake rate in 2,3-BD production, the *GAL10*, *GAL1*, *GAL7*, *PGM1* and *PGM2* genes involved in the Leloir pathway were overexpressed into the BD2M strain. These five consutructed strains were tested for a performance of galactose consumption in batch fermentation under oxygen-limited conditions. Only the strain for overexpression of the *PGM2* gene, the BD2M_PGM2 strain obtained 13% increased galactose uptake rate. Also, the BD2M_PGM2 strain resulted in 42% increased galactose uptake rate and 39% improved 2,3-BD productivity compared to the control strain, BD2M strain in a fed-batch fermentation.

Finally, to decrease the accumulation of glycerol and to solve the C_2-dependent growth in 2,3-BD-producing Pdc-deficient *S. cerevisiae*, the NADH oxidase (*noxE*) gene from *Lactococcus lactis* and the pyruvate

decarboxylase 1 (*pdc1*) gene from *Candida tropicalis* were expressed in the BD2M_PGM2 strain. To test for a performance of 2,3-BD production, batch and fed-batch fermentation was carried out. Unlike other conditions of fermentations in this study, this cultivation was conducted without addition of ethanol due to expression of the *pdc1* gene. The resulting strain (BD2M_PGM2_Ctnox) obtained 19% reduced yield of glycerol and was able to grow without addition of ethanol in a batch fermentation. Also, in a fed-batch fermentation, the resulting strain produced 116 g/L of 2,3-BD from glucose and galactose with a low glycerol yield (0.08 $g_{glyerol}/g_{sugars}$) with 0.47 $g_{2,3\text{-}BD}/g_{sugars}$ of yield and 1.75 g/L/h of productivity in a fed-batch fermentation. These results suggested that the BD2M_PGM2_Ctnox strain is suitable for producing 2,3-BD from marine biomass for industrial applications

Keywords : 2,3-butanediol, pyruvate decarboxylase (Pdc), galactose, marine biomass, mixed sugar fermentation, mth1, phosphoglucomutase (Pgm2), catabolite repression, redox balance

Student Number: 2014-20591

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I. INTRODUCTION

1. 2,3-Butanediol

2,3-Butanediol (2,3-BD) is a chiral compound and also known as 2,3-butylene glycol, 2,3-dihydroxybutane, or dimethylethlene glycol. The chemical formula of 2,3-BD is $CH_3CH(OH)CH(OH)CH_3$ with a molecular weight of 90.12 kDa and has three stereoisomeric forms: *D*-(-)-, *L*-(+)- and *meso*- (**Figure 1**) (Syu *et al.* 2001).

2,3-BD is used as an antifreeze agent due to its low freezing point of -60℃ (Celinska *et al.* 2009). Especially, 2,3-BD is a promising platform chemical with wide applications in the chemical industry since it can be converted into other valuable chemicals by dehydration, dehydrogenation, ketalization and esterification. 1,3-Butadiene, a dehydration product of 2,3-BD, is a main substance used for producing synthetic rubber, and is one of the primary building blocks in the renewable chemical industry (Christensen *et al.* 2008; Syu *et al.* 2001). More compounds can be synthesized from 2,3-BD as a precursor, such as methyl ethyl ketone (MEK) used in liquid fuel additives and polyurethane used in cosmetic products and drugs (Christensen *et al.* 2008; Garg *et al.* 1995). In addition, acetoin and diacetyl which is the

dehydrogenation products of 2,3-BD are also used as flavoring agents, food products and cosmetics (Bartowsky *et al.* 2004; Garg *et al.* 1995). The ketalization of 2,3-BD produces acetone 2,3-BD ketal, which is a potential gasoline blending agent. Esterified 2,3-BD can be utilized as a precursor for cosmetics, drugs and plasticizers (Garg *et al.* 1995).

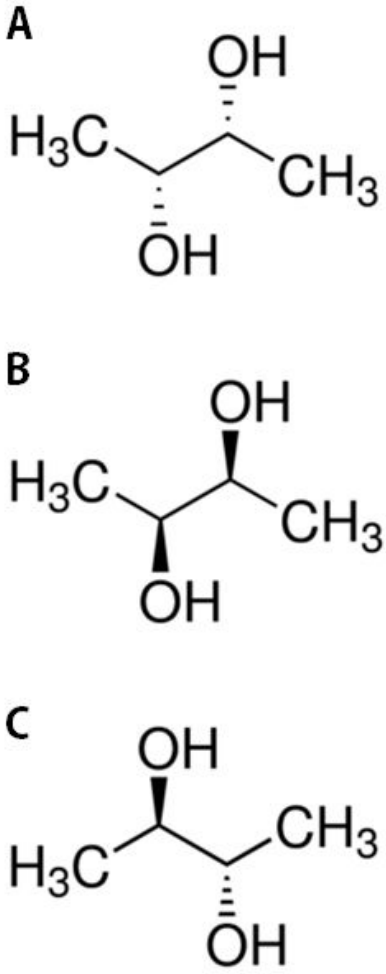

Figure 1. Stereoisomers of 2,3-BD.

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

2. Marine biomass as feedstock

With growing attention of global warming and support for sustainable energy, efforts for the production of bio-based chemicals and biofuels from renewable biomass have attracted attention (Serrano-Ruiz *et al.* 2010). Biomass has a short carbon cycle since it can be generated at least once in several years. In addition, the use of biomass can solve some environmental problems because biomass also includes industrial wastes.

Biomass can be divided into three types according to raw materials used. The first-generation biomass is starchy feedstocks such as corn and wheat. Its use causes an ethical problem because it is closely related to food-crop and was blamed for a major cause for the food crisis. The second-generation biomass is non-food terrestrial plants, called lignocellulosic biomass. It should be treated to liberate cellulose and hemicellulose for delignification and requires arable lands. The third-generation biomass is aimed to utilize macroalgae as an alternative resource. Macroalgae, so-called seaweeds, is regarded as a viable alternative renewable feedstock to starch-based and lignocellulosic biomass because, in contrast to lignocellulosic biomass, seaweed biomass can be easily hydrolyzed into monomeric sugars.

Furthermore, marine biomass does not require arable lands whereas terrestrial biomass production would compete for arable land with food crops (Wei *et al.* 2013). Macroalgaes are generally classified into three groups, green, brown and red algae according to the colors of photosynthetic pigments in the cells. Macroalgae consists of various carbohydrates depending on their species. (Park *et al*. 2011). Among them, red seaweeds contain high content of glucose and galactose (Wei *et al*. 2013). Therefore, efficient utilization of both glucose and galactose is important for microbial production of biofuels and bio-based chemicals from red seaweed hydrolysates in an economical manner.

In a wild type *Saccharomyces cerevisiae*, synthesis of galactose-metabolizing enzymes is under tight regulation in the presence of glucose by a mechanism called catabolite repression. As a result, the yeast utilizes galactose only after glucose is depleted when both sugars are concurrently present in the medium (Lee *et al.* 2011; Bae *et al.* 2014). The sequential consumption of mixed sugars and diauxic growth results in the reduction of the overall productivity (Kim *et al.* 2012). Therefore, simultaneous consumption of glucose and galactose is necessary for the improvement of microbial fermentation processes.

3. Galactose metabolism in *S. cerevisiae* in glucose and galactose mixed fermentation

In *S. cerevisiae*, galactose is metabolized via the Leloir pathway. The Leloir pathway is necessary because the initial enzymes of glycolysis are unable to recognize galactose. This pathway includes five enzymes to convert galactose to glucose-6-phosphate. These five enzyme activities are provided by five proteins: galactose mutarotase (Gal10), galactokinase (Gal1), galactose-1-phohsphate uridyltransferase (Gal7), UDP-galactose-4-epimerase (Gal10) and phosphoglucomutase (Pgm1 and Pgm2; minor and major isoform, respectively), where Gal10 exerts dual functions (**Figure 2**) (Timson *et al.* 2007). The genes coding for the Leloir proteins, hereafter called the *GAL* genes, are subjected to tight transcriptional regulation (Ostergaard *et al.* 2000; Zaman *et al.* 2008). In presence of glucose, Mig1 and Hxk2 in the nucleus of *S. cerevisiae* lead to transcriptional repression of the *GAL* genes. The overall regulatory mechanism is illustrated in **Figure 3.**

Several studies have been reported to enhance galactose utilization in *S. cerevisiae*. Through deletion of three negative regulators of Gal6, Gal80 and Mig1, specific galactose uptake rate increased by 41%

compared with a wild type strain (Ostergaard *et al.* 2000). In addition, overexpression of *PGM2* encoding phosphoglucomatse 2 in the Leloir pathway reduced the lag phase and enhanced growth rate in galactose medium (Sanchez *et al.* 2010). Further, to improve galactose utilization in mixed sugar fermentation, manipulation of the genes involved in catabolite repression has been investigated. Overexpression of the truncated *TUP1* gene encoding a general transcription repressor alleviated glucose repression and resulted in a 2.5-fold higher galactose consumption rate than the control strain (Lee *et al.* 2011). Furthermore, deletion of the hexokinase and glucose signaling protein *HXK2* allowed *S. cerevisiae* to consume galactose without a lag phase under oxygen-limited conditions. However, glucose and galactose were consumed sequentially and galactose consumption was limited because catabolite repression still existed in the engineered *S. cerevisiae* strains (Bae *et al.* 2014).

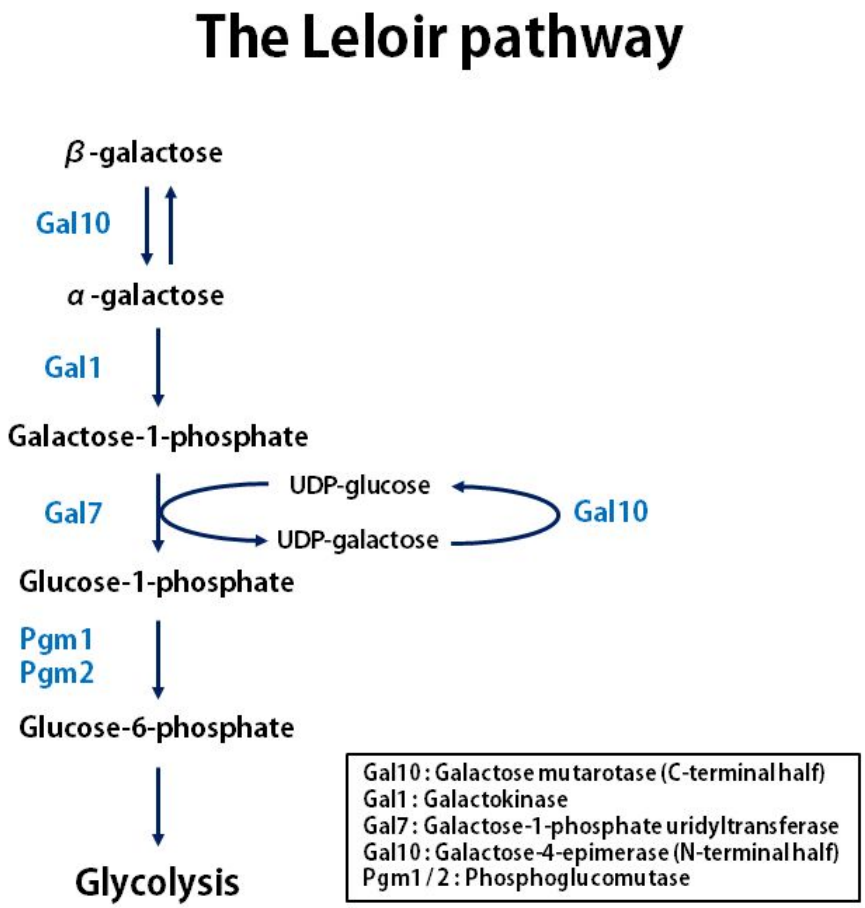

Figure 2. Galactose metabolism in *S. cerevisiae* via the Leloir pathway

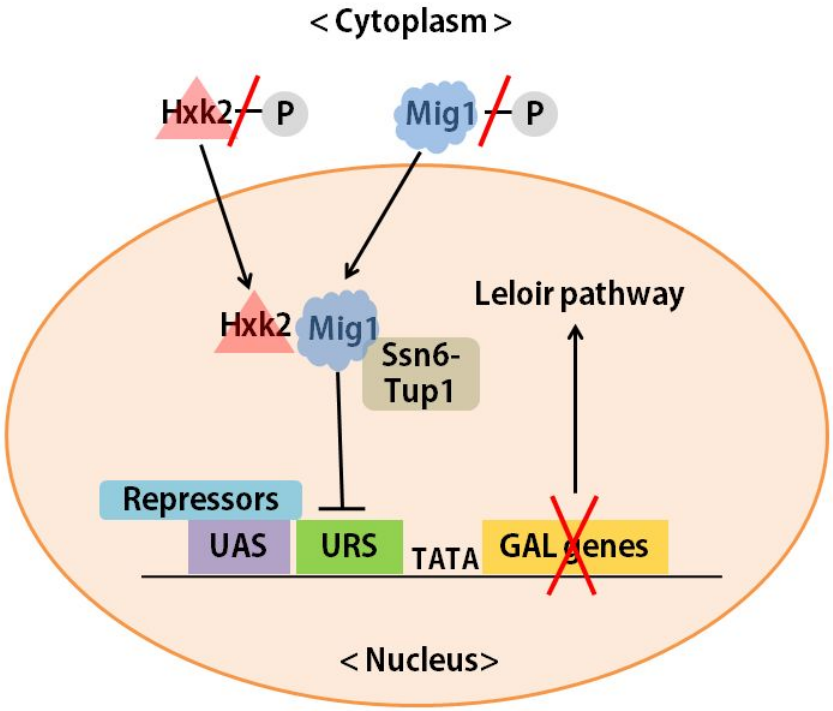

Figure 3. Mechanism of catabolite repression in *S. cerevisiae*. The repressors binding to UAS sequence and Mig1 binding to URS sequence along with general co-repressor Ssn6-Tup1 allows the yeast to tightly regulate the transcription of the *GAL* genes.

4. 2,3-BD production using bacteria

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, *Klebsiella pneumoniae*, *K. oxytoca* and *Paenibacillus polymyxa* are unbeatable in the efficient production of 2,3-BD with high yield and productivity. Also, *Enterobacter aerogenes* and *Serratia marcescens* are considered promising microorganisms (**Table 1**) (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. As shown in **Figure 4**, pyruvate from glycolysis can be converted into α -acetolactate catalyzed 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 convert diacetyl to acetoin. Finally, acetoin can be reduced to 2,3-BD by acetoin reductase (AR), also known as butanediol dehydrogenase (BDH) (Celinska *et al.* 2009).

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

Table 1. Microbial 2,3-BD production.

Strain	Substrate	Method	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
Klebsiella pneumoniae	Glucose	Fed-batch	150.0	0.43	4.21	Ma *et al.* (2009)
Klebsiella oxytoca	Glucose	Fed-batch	130.0	0.47	1.62	Ji *et al.* (2010)
Paenibacillus polymyxa	Sucrose	Fed-batch	111.0	-	2.05	Hassler *et al.* (2012)
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)
S. cerevisiae	Glucose	Fed-batch	96.2	0.28	0.39	Kim *et al.* (2013)
S. cerevisiae	Glucose, Galactose	Fed-batch	100.0	-	0.33	Lian *et al.* (2014)

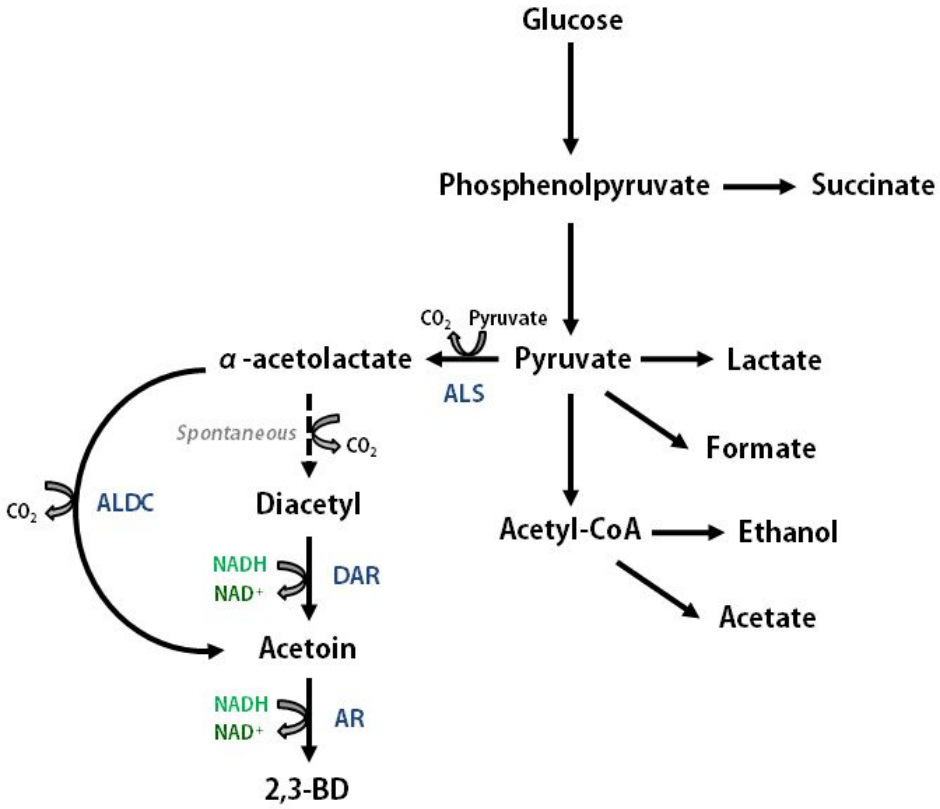

Figure 4. Mixed acid-2,3-BD pathway in bacteria. The names of enzymes are abbreviated as follows: ALS (α–acetolactate synthase), ALDC (α–acetolactate decarboxylase), DAR (Diacetyl reductase) and AR (Acetoin reductase).

5. 2,3-BD production using *S. cerevisiae*

S. cerevisiae produces trace amounts of 2,3-BD naturally, but the yield and productivity of 2,3-BD are very low (Celinska *et al.* 2009; Garg *et al.* 1995).

In *S. cerevisiae*, there are two pathways to synthesize 2,3-BD: One pathway via α–acetolactate and diacetyl and the other pathway via pyruvate or acetaldehyde (**Figure 5**). Pyruvate is converted into α–acetolactate by α–acetolactate synthase (*ILV2*) included in the valine and isoleucine synthetic pathways. Because acetolactate decarboxylase is not present in *S. cerevisiae* unlike 2,3-BD producing bacteria species, α–acetolactate cannot be enzymatically decarboxylated into acetoin. α–acetolactate can be converted into diacetyl by spontaneous decarboxylation in the presence of oxygen. Sequentially, diacetyl is reduced into acetoin by diacetyl reductase. Finally, acetoin is converted into 2,3-BD by butanediol dehydrogenase (*BDH1*). (Guymon *et al.* 1967; Gonzalez *et al.* 2010). In general, a wild-type *S. cerevisiae* produces 2,3-BD ranging from 0.4 to 2.0 g/L during wine fermentation (Ehsani *et al.* 2009).

2,3-BD production by wild-type *S. cerevisiae* has not been done extensively. Most previous researches are on improving a flavor in

beer and wine fermentations rather than producing 2,3-BD as a main product. Diacetyl and acetoin is undesirable due to their unpleasant flavor. Therefore, there have been many researches for reducing diacetyl and acetoin produced in a brewing process. Some studies succesfully reduced the diacetyl production by expression heterologous α–acetolactate decarboxylase from *K. terrigena* and *E. aerogenes*, resulting in an increase in 2,3-BD production (Suihko *et al.* 1990; Blomqvist *et al.* 1992). Also, overexpression of the *BDH1* gene contributed to an enhancement of 2,3-BD production by facilitating the conversion of acetoin into 2,3-BD. Recently, research on 2,3-BD production by engineered *S. cerevisiae* was reported. A strategy for gene deletion was done by using *in silico* genome scale metabolic analysis (Ng *et al.* 2012). Deletion of *ADH1*, *ADH3* and *ADH5* genes coding for alcohol dehydrogenase resulted in an improvement of 2,3-BD production by a 55-fold with reduced ethanol production compared to the wild type strain. The highest 2,3-BD titer (2.29 g/L) and yield (0.113 g 2,3-BD/g glucose) were obtained by the engineered *S. cerevisiae* deficient in the *ADH* genes in anaerobic conditions (Ng *et al.* 2012).

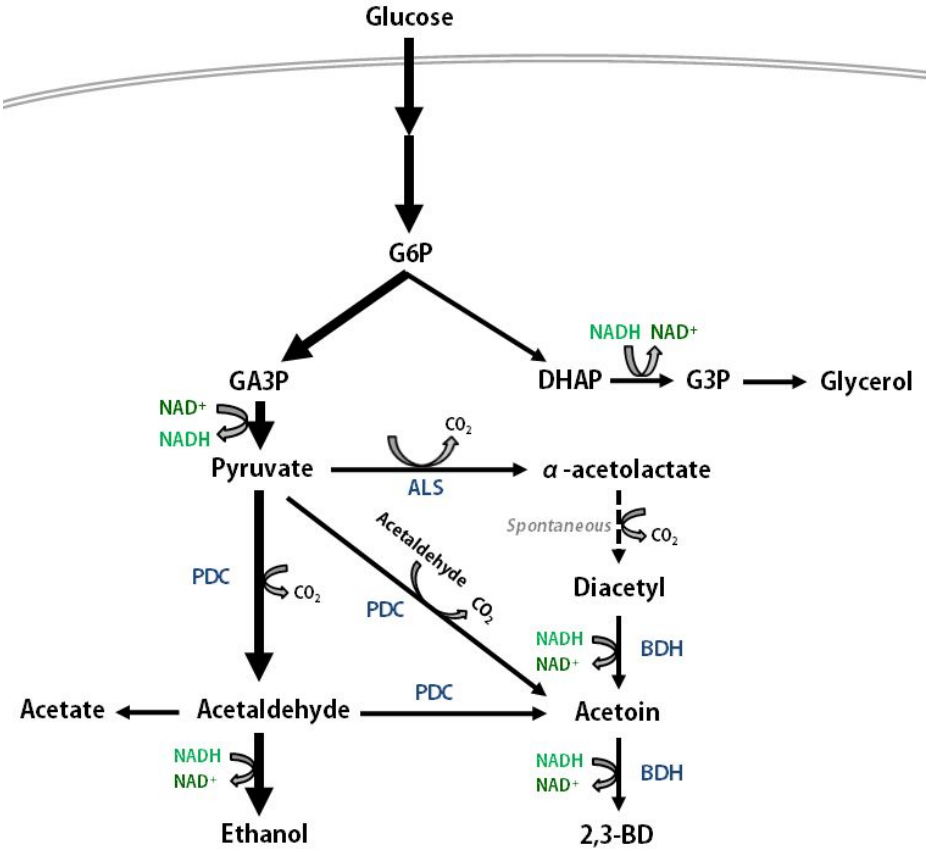

Figure 5. 2,3-BD biosynthetic pathway in *S. cerevisiae*. The name of enzymes are abbreviated as follows: ALS (α–acetolactate synthase), PDC (Pyruvate decarboxylase) and BDH (2,3-BD dehydrogenase)

6. 2,3-BD production using pyruvate decarboxylase-deficient *S. cerevisiae*

S. cerevisiae which has been traditionally used to ferment sugars to ethanol is a suitable host for the production of therapeutic proteins or chemicals by metabolic engineering. It is necessary to redirect carbon fluxes away from ethanol production to desired products because the yield of desired products should be maximized for economical production. To block the production of ethanol, a main fermentation product of *S. cerevisiae*, pyruvate decarboxylase (Pdc) was deleted in *S. cerevisiae.* The Pdc-deficient strains have been used for the production of 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. *S. cerevisiae* have three structural genes (*PDC1*, *PDC5* and *PDC6*) encoding active pyruvate decarboxylase isoenzymes. Among them, disruption of *PDC1* and *PDC5* or all three *PDC* gene 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 mutants need external supplement of two-carbon compounds such as acetate or ethanol for synthesis of cytosolic acetyl-CoA which is required to synthesize lysine and fatty acids (Flikweert *et al.* 1996; Pronk *et al.*1996). Cytosolic acetyl-CoA is synthesized from acetaldehyde in the cells. However, synthesis of acetaldehyde is blocked because of the elimination of Pdc activity, which leads to the shortage of cytosolic acetyl-CoA. Secondly, the Pdc-deficient mutants showed lower growth rate in 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, the Pdc-deficient strains suffer from redox imbalance because glucose represses respiration.

In the previous study, the *PDC1* and *PDC5* genes were deleted in the *S. cerevisiae* D452-2 strain (SOS2). Also, a C_2-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 change from alanine to proline (**Figure 6**).

Without extracellular glucose, Mth1 represses the transcription of the *HXT* genes with a transcriptional regulator of Rgt1. However, extracellular glucose generates an intracellular signal through the transmembrane glucose sensors Snf3 and Rgt2, which then activate the membrane-bound casein kinase I (Yck1/Yck2) The activated casein kinase I then promotes degradation of Mth1 by serial phosphorylation and ubiquitination. Degradation of Mth1 allows the de-repression of the *HXT* genes by inactivating the transcriptional regulator Rgt1, resulting in an enhancement of glucose uptake rate (Moriya *et al.* 2004; Lafuente *et al*. 2000) (**Figure 7**). The point mutation of the *MTH1* gene in the SOS4 strains was likely to reduce glucose uptake rate, which alleviates the pyruvate accumulation and redox imbalance from eliminating Pdc activity in the evolved Pdc-deficient strains (Kim *et al.* 2013).

In order to produce 2,3-BD in the engineered *S. cerevisiae* (SOS4) strain, the *alsS* and *alsD* genes coding for α–acetolactate synthase and α–acetolactate decarboxylase from *B. subtilis* were introduced. Additionally, the endogeneous *BDH1* gene coding for 2,3-BD dehydrogenase was overexpressed in the SOS4 strain. Therefore, the

resulting strain (BD4) produced 2,3-BD from glucose without ethanol production. The BD4 strain produced 96.2 g/L of 2,3-BD from glucose with a yield (0.28 g 2,3-BD/g glucose) and a productivity (0.39 g 2,3-BD/L·h) in a fed-batch fermentation. However, substantial amounts of glycerol were produced in parallel with 2,3-BD formation due to redox imbalance by excess production of cytosolic NADH under oxygen-limited conditions (Kim *et al.* 2013).

There have been numerous attempts to alter cytosolic concentration of NADH in *S. cerevisiae* through interconverting between NADH and NADPH, or by using the accumulation of metabolites capable of being reduced or oxidized (Nissen *et al.* 2000; Verho *et al.* 2003; Bro *et al.* 2006). Recently, research on reducing glycerol while increasing 2,3-BD production in the 2,3-BD-producing Pdc-deficient *S. cerevisiae* by expression of *Lactococcus lactis* NADH oxidase was reported (Kim *et al.* 2015). Because expression of the NADH oxidase led to a decrease in intracellular NADH concentration and NADH/NAD^+ ratio, accumulation of glycerol was reduced in the Pdc-deficient *S. cerevisiae*.

Also, in order to solve the defect of the Pdc-deficient strains which is two-carbon compound dependent, a Ph. D. thesis was reported recently. The introduction 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* (Kim *thesis.* 2016).

In addition to glucose, 2,3-BD production form cellulosic sugars, such as xylose and cellobiose by the engineered yeast have been reported (Kim *et al.* 2014; Nan *et al.* 2014). These researches suggested the possibility of producing 2,3-BD sustainably and efficiently from cellulosic sugars.

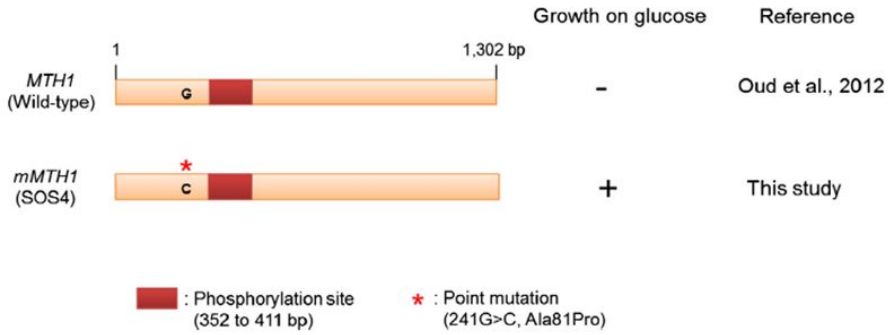

Figure 6. Comparison of the isolated mutant allele of *MTH1*gene from the Pdc-deficient mutants (SOS4) exhibiting glucose tolerance (Kim *et al.* 2013).

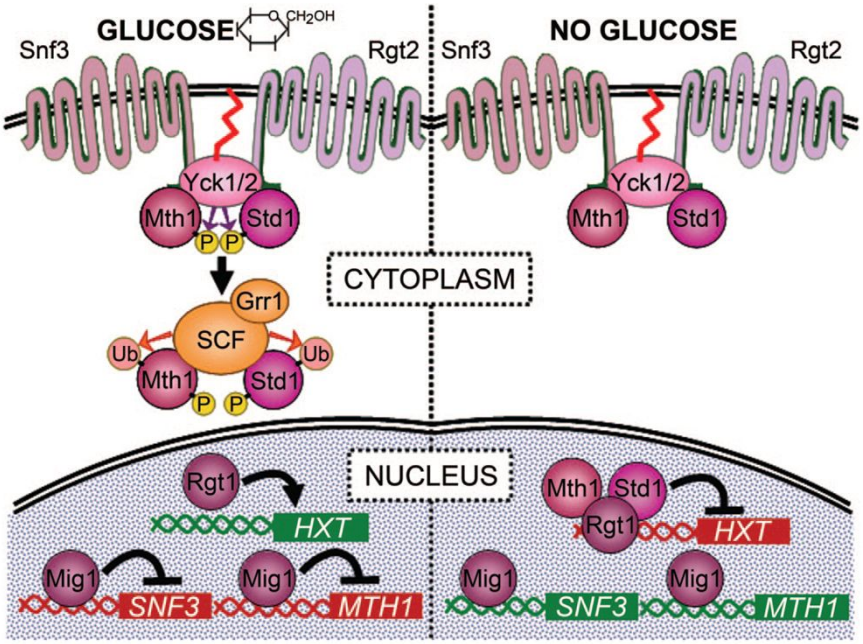

Figure 7. The Snf3/Rgt2 signaling pathway (Santangelo *et al.* 2006).

7. Research objectives

This study was focused on the production of 2,3-BD from a mixture of glucose and galactose by engineered *S. cerevisiae.* The specific objectives of this research are listed:

1) Production of 2,3-BD by simultaneous consumption of glucose and galactose

2) Increase of galactose uptake rate by overexpression of the *GAL* genes involved in the Leloir pathway

3) Improvement of 2,3-BD production by modulation of NADH metabolism and Pdc expression levels in Pdc-deficient *S. cerevisiae*

Ⅱ. Materials and Methods

1. Reagents

All chemicals used were of reagent grade. Glucose, galactose, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid) 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 purchased from Difco (Detroit, MD., 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

Strains used in this study are listed in **Table 2**. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used for gene cloning and manipulation. *S. cerevisiae* D452-2 (*Matα, leu2, his3, ura3, can1*) (Hosaka *et al.* 1992) and the Pdc-deficient *S. cerevisiae* D452-2 strain (SOS2) was used as host strains for the construction of the engineered strains. The deletion of both *PDC1* and *PDC5* resulted in complete loss of Pdc activity (Hohmann *et al.* 1990; Flikweert *et al.* 1996)

The SOS2 and SOS2_Mth1 were constructed by Dr. S. J. Kim at Seoul National University in Korea (Kim *et al.* 2013) and other strains in **Table 2** were constructed in this study. For the construction of the *MTH1* mutant strain (SOS2_Mth1), a single point mutation was created using the PCR-based allele replacement method as previously reported (Erdeniz *et al.* 1997). The point mutated *MTH1* gene was amplified from the SOS4 strain which was evolved to be able to grow in glucose medium (Kim *et al.* 2013).

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

Table 2. List of strains used in this study.

Name	Description	Reference
D452-2	*S. cerevisiae* Matα leu2 his3 ura3 can1	(Hosaka *et al.* 1992)
SOS2	D452-2 *pdc1*Δ *pdc5*Δ	(Kim *et al.* 2013)
SOS4	SOS2, evolved in excess glucose	(Kim *et al.* 2013)
SOS2_Mth1	SOS2, *MTH1*^{G241C}	(Kim *et al.* 2013)
BD4	SOS4, p426_alsS, p423_alsD and p425_BDH1	(Kim *et al.* 2013)
BD2	SOS2, p423_alsSalsD and p425_BDH1	In this study
BD2M	SOS2_Mth1, p423_alsSalsD and p425_BDH1	In this study
BD2M_GAL10	BD2M, p426_GAL10	In this study
BD2M_GAL1	BD2M, p426_GAL1	In this study
BD2M_GAL7	BD2M, p426_GAL7	In this study
BD2M_PGM1	BD2M, p426_PGM1	In this study
BD2M_PGM2	BD2M, p426_PGM2	In this study
BD2M_PGM2_Ctnox	BD2M_PGM2, p414_Ctnox	In this study

2.2. Plasmids

Four plasmids were used as mother vectors (**Figure 8**). For the introduction of the 2,3-BD biosynthetic pathway in Pdc-deficient *S. cerevisiae*, acetolactate synthase (*alsS*) and acetolactate decarboxylase (*alsD)* from *B. subtilis* and endogenous 2,3-BD dehydrogenase (*BDH1*) were expressed under the control of the *S. cerevisiae GPD* promoter and *CYC1* terminator. Five plasmids (p426GPD_GAL10, p426GPD_GAL1, p426GPD_GAL7, p426GPD_PGM1 and p426GPD_PGM2) containing the *GAL10*, *GAL1*, *GAL7*, *PGM1* and *PGM2* genes from *S. cerevisiae* under the control of a constitutive *GPD* promoter and *CYC1* terminator were introduced into the BD2M strain for overexpression of the *GAL* genes involved in the Leloir pathway. Also, pRS414AUR which is a chromosomal intergrating vector with the mutant AUR1-C gene derived from *S. cerevisiae* that confers aureobasidin A-resistance on cells was used for expression of the *noxE* gene from *L. lactis* and the *pdc1* gene from *C. tropicalis* (**Table 3**). There are the oligonucleotide sequence of primers used for cloning (**Table 4**). Abbreviations and significations used in this study are as follows. M

is mutation of the *MTH1* gene. Ctnox means expression of the *noxE* gene from *L. lactis* and the *pdc1* gene from *C. tropicalis*.

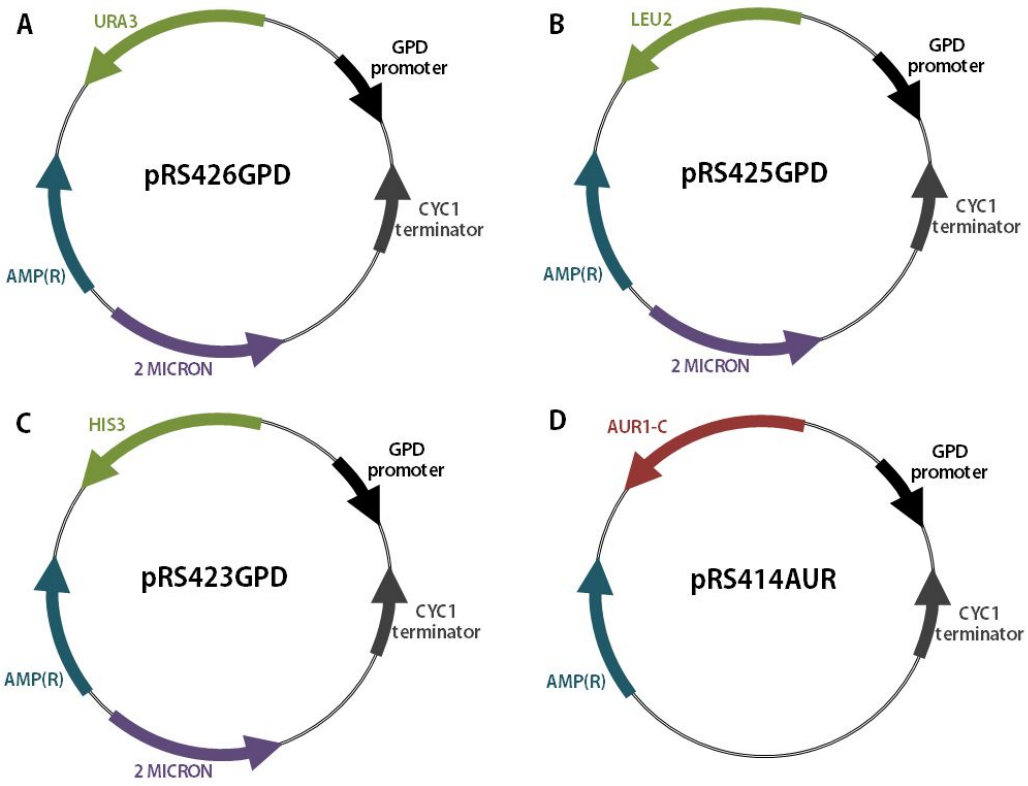

Figure 8. Mother vectors used in this study.

(A) pRS426GPD, (B) pRS425GPD, (C) pRS423GPD, (D) pRS414AUR

Table 3. 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, GPD* promoter, *CYC1* terminator, Amp^r	(Kim *thesis.* 2016)
p423_alsSalsD	pRS423GPD harboring *alsS* and *alsD* gene from *Bacillus subtilis* str.168	(Kim *et al.* 2014)
p425_BDH1	pRS425GPD harboring *BDH1* gene from *S. cerevisiae* D452-2	(Kim *et al.* 2013)
p426_GAL10	pRS426GPD harboring *GAL10* gene from *S. cerevisiae* D452-2	In this study
p426_GAL1	pRS426GPD harboring *GAL1* gene from *S. cerevisiae* D452-2	In this study
p426_GAL7	pRS426GPD harboring *GAL7* gene from *S. cerevisiae* D452-2	In this study
p426_PGM1	pRS426GPD harboring *PGM1* gene from *S. cerevisiae* D452-2	In this study
p426_PGM2	pRS426GPD harboring *PGM2* gene from *S. cerevisiae* D452-2	In this study
p414_Ctnox	pRS414AUR harboring *noxE* gene from *Lactococcus lactis* subsp. *cremonis* MG1363 and $GPD2_p$-*pdc1* from *Candida tropicalis* MYA3404-$CYC1_t$ cassette	In this study

Table 4. List of oligonucleotide used in this study.

Primer name	Oligonucleotide seuquence (5’ → 3’)
F_GPDp_seq	GTAGGTATTGATTGTAATTCTGTAAAT
R_CYCt_seq	ATAACTATAAAAAAATAAATAGGGAC
F_BamHI_GAL10	CGCGGATCCATGACAGCTCAGTTACAAAGTGAAAGT
R_XhoI_GAL10	CCGCTCGAGTCAGGAAAATCTGTAGACAATCTTGG
F_BamHI_GAL1	CGCGGATCCATGACTAAATCTCATTCAGAAGAAGTG
R_XhoI_GAL1	CCGCTCGAGTTATAATTCATATAGACAGCTGCCCA
F_BamHI_GAL7	CGCGGATCCATGACTGCTGAAGAATTTGATTTTTCTAG
R_XhoI_GAL7	CCGCTCGAGTTACAGTCTTTGTAGATAATGAATCTGAC
F_BamHI_PGM1	CGCGGATCCATGTCACTTCTAATAGATTCTGTACCAAC
R_XhoI_PGM1	CCGCTCGAGCTATGTGCGGACTGTTGGTTC
F_BamHI_PGM2	CGCGGATCCATGTCATTTCAAATTGAAACGGTTCCC
R_XhoI_PGM2	CCGCTCGAGTTAAGTACGAACCGTTGGTTCTTCAG
F_GAL10_seq	GAGACGATTATGATTCCAGAGATGGTA
F_GAL1_seq	TGAAACCGCCCCAACCAACTATAATT
F_PGM1_seq	GAATCTAACCTATGCACGAACTCTTGT
F_GPD2p_seq	AAACGACATATCTATTATAGTGGGGAG
F_SacI_GPD2p	CGAGCTCCAAAAACGACATATCTATTATAGTG

R_CYC1t_SacI	CGAGCTCGGCCGCAAATTAAAGCC
F_PDC1_seq1	CTTTGGGTAGATTCTTCTTTGAAAG
F_PDC1_seq2	AAACCGGTACCTCTGCTTTCGG

3. DNA manipulation and transformation

3.1. Enzymes

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

3.2. Polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) was performed with the Accupower™ PCR PreMix (Bioneer Co., Daejon, Korea) in the 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 (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 performed by SolGent (Daejeon, Korea).

3.6. 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. Ethanol 0.5 g/L was added if necessary. 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 (10 g/L yeast extract and 20 g/L bacto-peptone) and YNB medium which lacked appropriate amino acids were used for selection of 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 for cultivation of yeast strains.

4.2. Batch fermentations.

All cultures were carried out at 30°C. Seed-cultures and pre-cultures of yeast cells were conducted aerobically in 5 mL test tubes and 250 mL baffled flasks respectively. Main flask batch cultures were conducted 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 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 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 a mixture of glucose and galactose. The ratios of glucose and galactose in the cultiavation vary. For evaluating carbon consumption rates with different glucose:galactose ratios, 0,25,50,75 and 100 g/L of glucose were combined with 50 g/L of galactose, or 0,25,50,75 and 100 g/L of galactose were combined with 50 g/L glucose. Main cultures were carried out in 250 mL flasks with a working volume of 50 mL at 80 rpm under oxygen-limited conditions.

4.3. Fed-batch fermentations

To obtain high concentrations of 2,3-BD, fed-batch fermentation

was carried out using a 1 L bench-top fermentor (FERMENTEC, Korea) according to the method of the previous study with some modifications (Kim *et al.* 2015). 500 mL of YP medium with a mixture of glucose and galactose was used and pH was adjusted to 5.5 by the addition of 2N HCl or 2N NaOH intermittently. 200 rpm of agitation speed and 1.0 vvm aeration were maintained throughout the cultivation. For the cultivation of BD2M_Ctnox containing the *noxE* gene, agitation and aeration were changed during the cultiavation: from 500 rpm, 2.0 vvm to 200 rpm, 1.0 vvm according to the method of the previous research (Kim *thesis*. 2016).

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 250 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 9000 rpm for 5 min and washed in 5 mL of sterilized DDW and inoculated into a bioreactor with an initial OD_{600} of 10.0. After depletion of glucose and galactose, additional glucose and galactose solutions were added.

5. Analysis

5.1. Dry cell weight

Cell growth was monitored by optical density at 600 nm (OD_{600}) using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Dry cell weight (DCW) was calculated from the pre-determined conversion factor: $0.2 g_{DCW}/OD_{600}$.

5.2. Metabolite detection

Concentrations of glucose, galactose, glycerol, acetate, acetoin, 2,3-BD and ethanol were determined by a high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series, Santa Clara, CA, U.S.A) equipped with a Rezex-ROA-organic acid column (Phenomenex, CA). The metabolites were detected by a refractive index (RI) detector. The column was heated at 60℃ and eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min.

III. RESULTS AND DISCUSSIONS

1. Construction of the 2,3-BD producing Pdc-deficient *S. cerevisiae* strain by simultaneous consumption of glucose and galactose

1.1. Confirmation the effect of mutant *MTH1* on co-consumption of glucose and galactose

In the previous study, the evolved Pdc-deficient *S. cerevisiae* strain was generated by serial cultivation in excess glucose medium, then the evolved 2,3-BD-producing strain (BD4) was constructed by introducing the 2,3-BD biosynthetic enzymes (Kim *et al.* 2013). Among the chromosomal mutations of the BD4 strains, a single nucleotide polymorphism (SNP) was found at the 241 bp position of the *MTH1* gene in the genomic DNA of the BD4 strain. When flask cultivation was carried out with the BD4 strain in a mixture of glucose and galactose, both sugars were simultaneously consumed as shown in **Figure 9**. Since Mth1p is a transcription factor that regulates hexose transport in *S. cerevisiae*, the mutation on *MTH1* allowed growth in glucose media by reducing glucose uptake in the Pdc-deficient *S. cerevisiae* strain (Oud *et al.* 2012; Kim *et al.* 2013).

From this, I hypothesized that the *MTH1* mutation might be related to the simultaneous consumption of glucose and galactose. Thus, to confirm the effect of mutant *MTH1* on co-consumption of glucose and galactose, the Pdc-deficient *S. cerevisiae* strain SOS2 and SOS2_Mth1 which has the mutation on *MTH1* constructed by Dr. S. J. Kim at Seoul National University in Korea (Kim *et al.* 2013) was engineered. The 2,3-BD producing BD2 and BD2M strains were constructed by the introduction of the 2,3-BD biosynthetic enzymes using the above plasmids (**Table 3**).

To evaluate if the *MTH1* mutation affects the fermentation performances in a mixture of glucose and galactose, flask batch cultivations of both BD2 and BD2M strains were carried out using a mixture of 75 g/L of glucose and 90 g/L of galactose (**Figure 10**). While the BD2 strain fermented glucose and galactose sequentially as observed for the wild type *S. cerevisiae* (Bae *et al*. 2014), the BD2M strain was able to co-ferment both sugars with similar consumption rates of glucose (1.03 $g_{glucose}$/L/h) and galactose (1.06 $g_{galactose}$/L/h). Simultaneous consumption of glucose and galactose in the BD2M strain increased overall substrate consumption rate (1.95 g_{sugars}/L/h) compared with the BD2 strain (1.45 g_{sugars}/L/h). The 2,3-

BD productivity of the BD2M strain (0.62 g/L/h) was substantially higher than that of the BD2 strain (0.45 g/L/h), but 2,3-BD yields were similar between the BD2 (0.31 $g_{2,3\text{-BD}}/g_{substrate}$) and the BD2M (0.32 $g_{2,3\text{-BD}}/g_{substrate}$) strains (**Table 5**).

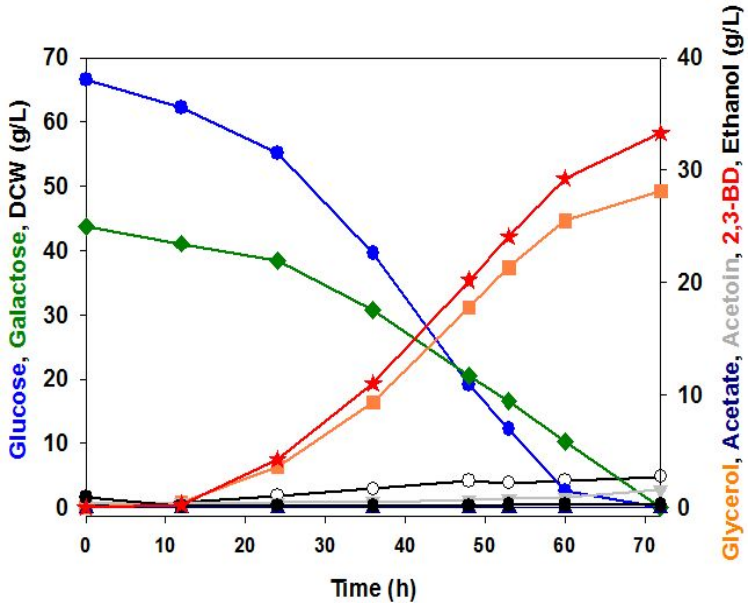

Figure 9. Batch fermentation profiles of BD4 strain cultured in a mixture of glucose and galactose.

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

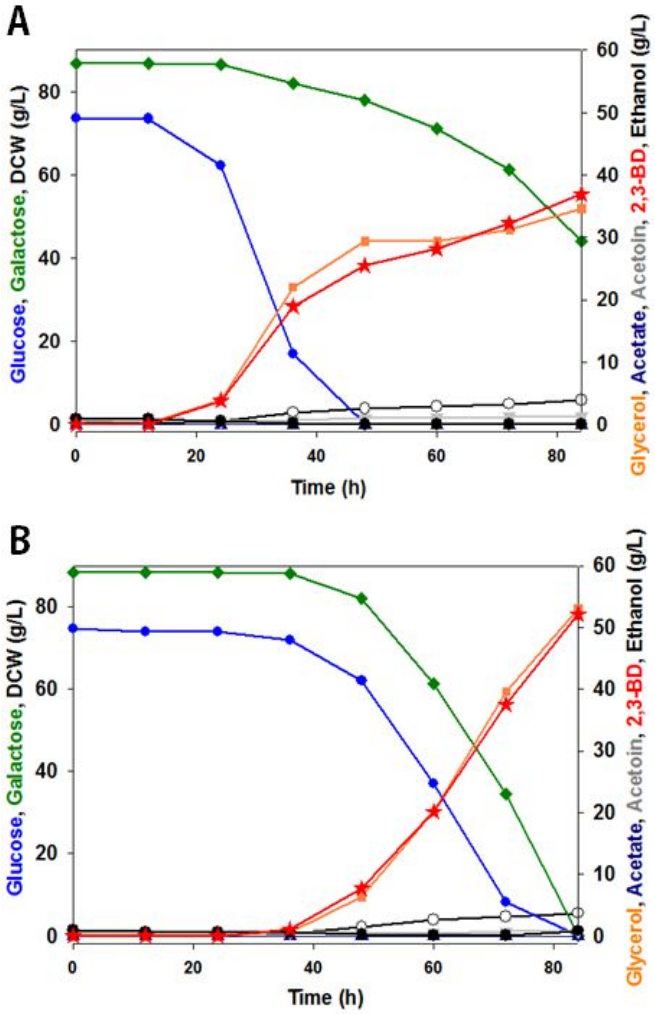

Figure 10. Batch fermentation profiles of the (A) BD2 and (B) BD2M strains cultured in a mixture of glucose and galactose.

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

Table 5. Summary of batch fermentation in part 1.1.

Strain	DCW (g/L)	Glucose uptake rate (g/L/h)	Glactose uptake rate (g/L/h)	Glycerol (g/L)	2,3-BD (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD2	5.8	1.53	0.51	34.6	36.9	0.31	0.44
BD2M	5.6	0.89	1.05	53.1	52.1	0.32	0.62

1.2. Influence of glucose and galactose ratio on 2,3-BD fermentation by the BD2M strain

As reported recently, the expression of the genes responsible for galactose metabolism are regulated by the ratio of glucose and galactose rather than complete repression of galactose genes by glucose (Escalante *et al*. 2015). Thus, to explore the influence of sugar ratio on carbon consumption rate in 2,3-BD-producing Pdc-deficient *S. cerevisiae*, the batch cultivations were carried out by the BD2M strain with different ratios of glucose and galactose (**Figure 11**). The BD2M strain could consume both sugars simultaneously under all the tested conditions, however, the galactose consumption rates were substantially changed by different ratios of glucose and galactose. The galactose consumption rate decreased with increasing glucose concentration. The galactose consumption rate in galactose medium without glucose (1.28 g/L/h) was substantially reduced by the addition of 100 g/L of glucose (0.51 g/L/h). On the other hand, glucose consumption rates decreased only 19.1% by the addition of 100 g/L of galactose from 1.36 to 1.10 g/L/h. Glucose and galactose consumption rates were influenced by the ratio of glucose and

galactose in medium at exceeding glucose levels that could trigger catabolite repression. Thus, proper control of glucose and galactose ratio in medium is required for the efficient production of 2,3-BD from a mixture of glucose and galactose.

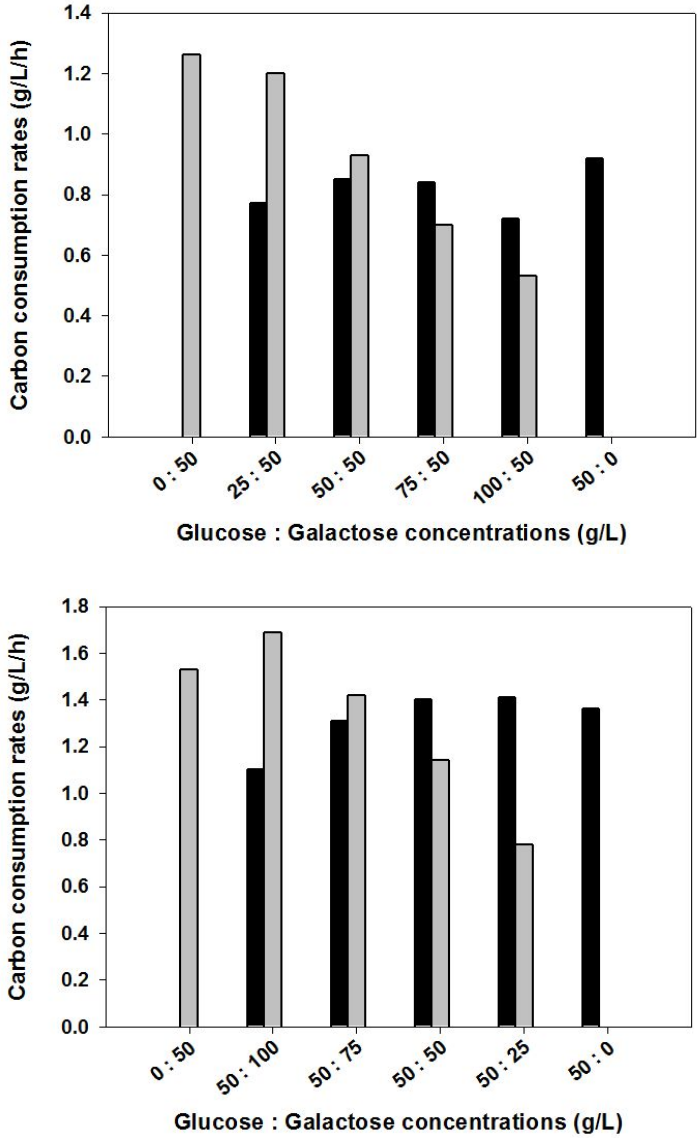

Figure 11. Carbon consumption rates of the BD2M strain according to different ratios of glucose and galactose.

Symbols : glucose consumption rate (black bar)

and galactose consumption rate (grey bar)

1.3. Fed-batch fermentation of the BD2M strain in glucose and galactose mixed sugars

In order to evaluate the fermentation behavior of the BD2M strain in a mixture of glucose and galactose, fed-batch fermentations were carried out with intermittent feeding of glucose and galactose (**Figure 12**). The initial fermentation medium contained 50 g/L of each glucose and galactose. After depletion of these carbon sources, additional glucose and galactose were added to the fermentation medium. The fed-batch fermentation with the BD2M strain resulted in 75.5 g/L of 2,3-BD in 138 hours of cultivation with a yield of 0.34 $g_{2,3\text{-BD}}/g_{sugars}$ along with substantial amounts of glycerol (45.3 g/L) as a by-product. Acetoin, a secondary by-product of 2,3-BD fermentation, also accumulated at less than 5.0 g/L (**Table 6**). In the rest of the fermentation, the galactose consumption rate was substantially reduced compared with glucose consumption rate. A possible explanation for this problem might be limited expression levels of galactose metabolic genes in the engineered strain during 2,3-BD fermentation.

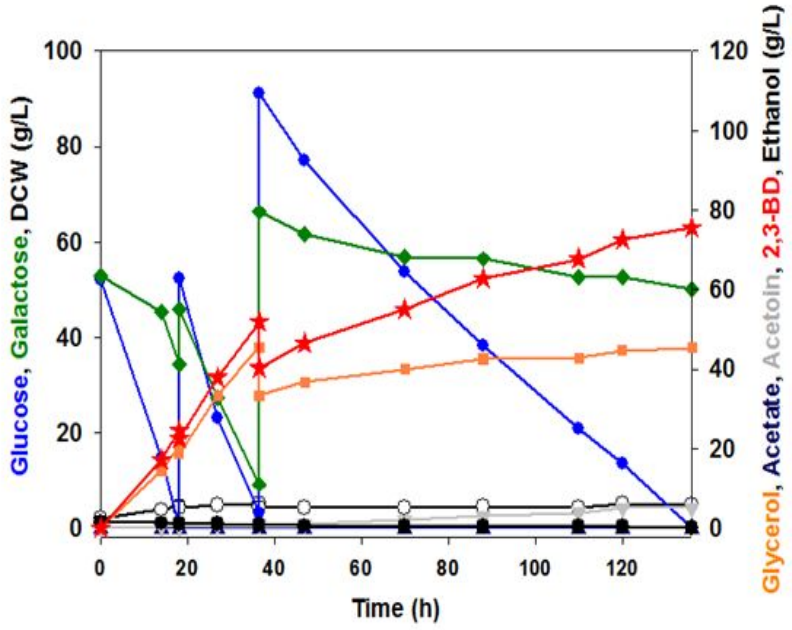

Figure 12. Fed-batch fermentation profiles of the BD2M strains cultured in a bioreactor.

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

Table 6. Summary of fed-batch fermentation in part 1.3

Strain	DCW (g/L)	2,3-BD (g/L)	Glycerol (g/L)	Acetoin (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD2M	5.2	75.5	45.3	4.9	0.23	0.34	0.56

2. Overexpression of galactose metabolic enzymes to increase galactose uptake rate in 2,3-BD production

According to the fed-batch fermentation of the BD2M strain in the previous part of this study, galactose consumption rate was substantially reduced compared with glucose consumption rate in the rest of the fermentation.

Several studies have been reported to enhance galactose utilization in *S. cerevisiae*. Through deletion of three negative regulators of Gal6, Gal80, and Mig1, specific galactose uptake rate increased by 41% compared with a wild type strain (Ostergaard *et al.* 2000). In addition, overexpression of *PGM2*, which encodes phosphoglucomutase 2 in the Leloir pathway, reduced the lag phase and enhanced growth rate in galactose medium (Sanchez *et al.* 2010). Further, to improve galactose utilization in mixed sugar fermentation, manipulation of the genes involved in catabolite repression has been investigated. Overexpression of the truncated *TUP1* gene, which encodes a general transcription repressor, alleviated glucose repression and resulted in a 2.5-fold higher galactose consumption rate than control strain (Lee *et al*. 2011). Furthermore, deletion of

the hexokinase and glucose signaling protein *HXK2* allowed *S. cerevisiae* to consume galactose without a lag period under oxygen-limited conditions. However, glucose and galactose were consumed sequentially and galactose consumption was limited because catabolite repression still existed in the engineered *S. cerevisiae* strain (Bae *et al.* 2014).

In this study, to increase the galactose uptake rate in 2,3-BD producing Pdc-deficient *S. cerevisiae*, the *GAL g*enes overexpressing BD2M_GAL10, BD2M_GAL1, BD2M_GAL7, BD2M_PGM1 and BD2M_PGM2 strains were constructed using the above plasmids (**Table 3**). Thus, to confirm the effect of overexpression of *GAL* genes on galactose consumption rate, flask batch cultivations were carried out. Among the constructed strains tested, the BD2M_PGM2 strain only improved galactose uptake rate (1.43 g/L/h) relative to the control strain, BD2M strain (1.27 g/L/h). The 2,3-BD productivity of BD2M_PGM2 also increased compared to the BD2M strain from 0.90 g/L/h to 0.98 g/L/h (**Table 7**).

In order to confirm the fermentation aspects of the BD2M_PGM2 strain in a mixture of glucose and galactose, fed-batch fermentation was carried out. Feeding of glucose and galactose was performed 4

times intermittently. The initial fermentation medium contained 50 g/L of glucose and galactose (**Figure 13**). The fed-batch fermentation with the BD2M_PGM2 strain resulted in 106.2 g/L of 2,3-BD in 138 hours of fermentation with a yield of 0.38 $g_{2,3\text{-}BD}/g_{sugars}$ and a productivity of 0.78 g/L/h along with 42% increased galactose uptake rate compared to BD2M strain (**Table 8**). This indicated that *PGM2* could be a key role in controlling the flux through the Leloir pathway.

Table 7. Summary of batch fermentation in part 2.

Strain	DCW (g/L)	Glucose uptake rate (g/L/h)	Glactose uptake rate (g/L/h)	Glycerol (g/L)	2,3-BD (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD2M	3.6	1.35	1.27	39.4	43.5	0.34	0.90
BD2M_GAL10	3.4	1.38	1.29	40.6	45.0	0.33	0.91
BD2M_GAL1	3.4	1.30	1.30	40.4	44.8	0.33	0.90
BD2M_GAL7	3.7	1.38	1.29	39.1	43.8	0.33	0.90
BD2M_PGM1	3.6	1.38	1.22	37.2	42.5	0.33	0.88
BD2M_PGM2	3.4	1.38	1.43	42.7	46.7	0.34	0.98

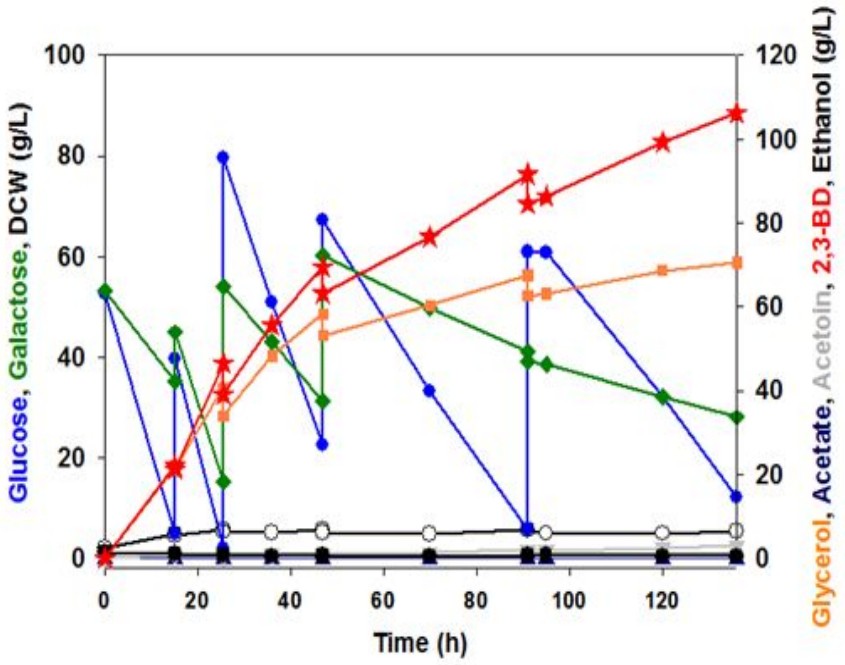

Figure 13. Fed-batch fermentation profiles of the BD2M_PGM2 strains cultured in a bioreactor.

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

Table 8. Summary of fed-batch fermentation in part 2.

Strain	DCW (g/L)	2,3-BD (g/L)	Glycerol (g/L)	Acetoin (g/L)	Galactose uptake rate (g/L/h)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD2M (in part 1.3)	5.2	75.5	45.3	4.9	0.52	0.23	0.34	0.56
BD2M_PGM2	5.7	106.2	70.5	3.0	0.74	0.25	0.38	0.78

3. Expression of *L. lactis noxE* and *C. tropicalis pdc1* in the Pdc-deficient *S. cerevisiae*

As reported, the Pdc-deficient strains have two kinds of potential defect. First thing is accumulation of glycerol because of redox imbalance. Several studies have been reported to decrease the intracellular NADH/NAD^+ ratio for redox balance (Heux *et al.* 2006; Vemuri *et al.* 2007). Recently, the *L. lactis* water-forming NADH oxidase gene (*noxE)* was expressed at five different levels to redirect the flux of NADH from glycerol to 2,3-BD through alteration of the NADH/NAD^+ ratio, resulting in 23.8% higher yield of 2,3-BD and 65.3% lower yield of glycerol than those of the isogenic strain without *noxE* (Kim *et al.* 2015). Second thing is a need for external supplement of two-carbon compounds for acetyl-CoA synthesis which is required to synthesize lysine and fatty acids (Flikweert *et al.* 1996). In the recent previous study, to construct the system of C_2-independent growth on the Pdc-deficient strain, an investigation of the *pdc* genes with low Pdc-activity and selection of fine-tuned Pdc-expression plasmids were conducted. The *pdc1* gene from *C. tropicalis* and the *GPD2* promoter were selected (Kim *thesis.* 2016).

As shown before, glycerol substantially accumulated and all cultivations were carried out with adding 0.5 g/L ethanol as a C_2-compound to support growth. Therefore, in order to solve these defects, expression of the *noxE* gene from *L. lactis* and the *pdc1* gene from *C. tropicalis* were employed using the above plasmids (**Table 3**). To confirm the effect of the *noxE gene* and the *pdc1* gene on Pdc-deficient strains, flask batch cultivations were carried out without ethanol addition (**Figure 14**). The BD2M_PGM2_Ctnox strain reduced glycerol yield (0.25 $g_{glycerol}/g_{sugars}$) by 19% compared with the control strain, BD2M_PGM2 strain (0.31 $g_{glycerol}/g_{sugars}$). Also, the yield and productivity of 2,3-BD were similar, resulting in overcoming C_2-dependent growth of Pdc-deficient strains (**Table 9**).

In order to evaluate the fermentation aspects of the BD2M_PGM2_Ctnox strain in a mixture of glucose and galactose, fed-batch fermentations were carried out without addition of ethanol. Feeding of glucose and galactose was conducted only one time. The initial fermentation medium contained 100 g/L of glucose and galactose. In consideration of the characteristics of the BD2M_PGM2_Ctnox strain containing the *noxE* gene, agitation and aeration were changed during the cultivation: from 500 rpm, 2.0

vvm to 200 rpm, 1.0 vvm according to the method of the previous research (Kim *thesis*. 2016) (**Figure 15**). The fed-batch fermentation with the BD2M_PGM2_Ctnox strain resulted in 115.7 g/L of 2,3-BD in 66 hours of cultivation with a yield of 0.47 $g_{2,3\text{-}BD}/g_{sugars}$ and a productivity of 1.75 g/L/h along with decreased yield of glycerol (0.08 $g_{glycerol}/g_{sugars}$) as a by-product (**Table 10**). The expression of *L. lactis noxE* and *C. tropicalis pdc1* could be an efficient strategy for metabolic engineering to produce fine chemicals. In addition, these results suggested that the BD2M_PGM2_Ctnox strain is suitable for producing 2,3-BD from marine biomass for industrial applications.

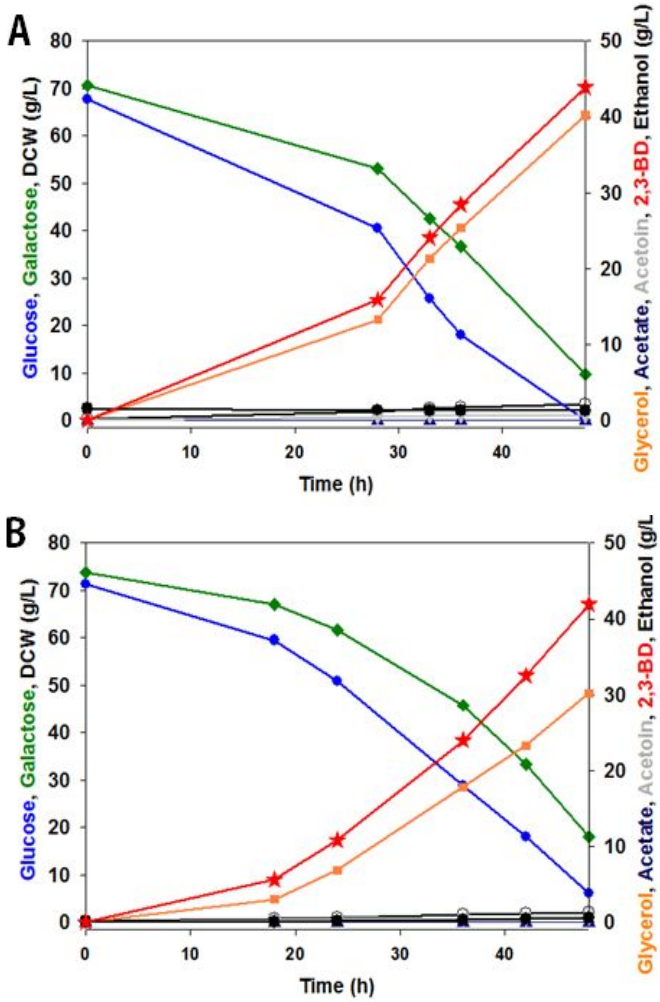

Figure 14. Batch fermentation profiles of the (A) BD2M_PGM2 (with 0.5 g/L ethanol) and (B) BD2M_PGM2_Ctnox (without ethanol) strains cultured in a mixture of glucose and galactose.

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

Table 9. Summary of batch fermentation in part 3.

Strain	DCW (g/L)	Glucose uptake rate (g/L/h)	Glactose uptake rate (g/L/h)	Glycerol (g/L)	Glycerol yield (g/g)	2,3-BD (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD2M_PGM2	3.4	1.38	1.25	40.2	0.31	43.4	0.34	0.89
BD2M_PGM2 _Ctnox	2.0	1.36	1.16	30.2	0.25	41.9	0.35	0.87

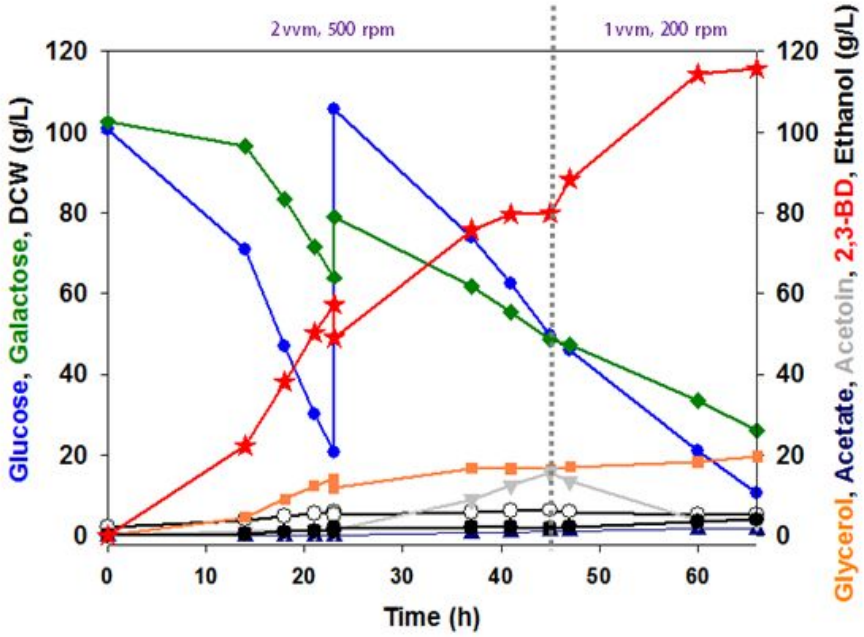

Figure 15. Fed-batch fermentation profiles of the BD2M_PGM2_Ctnox strain cultured in a bioreactor (without ethanol).

Grey dotted line is the time of changing the aeration and agitation from 2.0 vvm, 500 rpm to 1.0 vvm, 200 rpm.

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

Table 10. Summary of fed-batch fermentation in part 3.

Strain	DCW (g/L)	2,3-BD (g/L)	Glycerol (g/L)	Acetoin (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD2M_PGM2 _Ctnox	6.3	115.7	19.6	3.5	0.08	0.47	1.75

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) A single point mutation on the *MTH1* gene allowed *S. cerevisiae* to alleviate catabolite repression in glucose and galactose mixtures. The engineered 2,3-BD-producing *S. cerevisiae*, BD2M strain consumed glucose and galactose simultaneously and 2,3-BD was produced with higher productivity than the control strain, BD2 strain.

(2) To increase the galactose uptake rate in 2,3-BD-producing *S. cerevisiae*, the five *GAL* genes of the Leloir pathway were overexpressed in the BD2M strain. The strain for overexpression of the *PGM2* gene, the BD2M_PGM2 strain increased galactose uptake rate by 42% and improved productivity of 2,3-BD by 39% compared to the control strain, BD2M strain in a fed-batch fermentation.

(3) To decrease the accumulation of glycerol, a by-product and to solve the C_2-dependent growth of 2,3-BD-producing Pdc-deficient *S. cerevisiae* on glucose media, the *noxE* gene from *L. lactis* and the *pdc1* gene from *C. tropicalis* were expressed in the BD2M_PGM2 strain. The resulting BD2M_PGM2_Ctnox strain produced 115.7 g/L of 2,3-BD from glucose and galactose with 0.47 $g_{2,3\text{-BD}}/g_{sugars}$ of yield and 1.75 g/L/h of productivity, resulting in reduction of glycerol yield compared to BD2M strain in a fed-batch fermentation without addition of ethanol.

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국 문 초 록

2,3-Butanediol (2,3-BD)는 산업적 활용도가 높은 플랫폼 화학 소재이다. 2,3-BD는 화학적 생산이 가능하지만 유가 변동문제와 지구온난화 문제로 인해 최근 바이오 기술이 개발됨에 따라 생물공학적으로 생산이 주목 받고 있다. 2,3-BD의 생물공학적 생산에는 주로 박테리아가 사용되는데 고 수율로 2,3-BD를 생산할 수 있지만 이들 대부분이 병원성 박테리아로 분류되기 때문에 안전과 산업화 측면에서 대량 생산 공정 구축이 어렵다. 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 자연 상태에서는 2,3-BD를 거의 생산하지 못하고 에탄올을 주로 생산하기에 고효율의 2,3-BD를 생산하기 위해서는 대사공학적 방법을 이용한 재조합 *S. cerevisiae*의 구축이 요구된다.

또한, 경제적 측면으로 볼 때 해양 바이오매스로부터 2,3-BD를 생산하는 것이 2,3-BD의 지속 가능한 상업화를 위해 해결해야 할 주요 연구 과제이다. 하지만 *S. cerevisiae*는 해양 바이오매스에 다량 존재하는 포도당과 갈락토오스를 동시에 소모하지 못하고, 포도당이 모두 소모된 후에야 갈락토오스가 이용되는 포도당에 의한 전사 억제(catabolite repression)의 단점을 가진다.

선행연구자에 의해 *S. cerevisiae*의 주요 대사 산물인 에탄올 생성을 억제하고자 pyruvate decarboxylase 활성이 완전히 저해된 효모 (SOS2)와, SOS2 균주를 포도당 배지에서 생장이 가능하도록 하기 위해 이볼루션시킨 효모 (SOS4)를 제작한 바 있다. 이볼루션의 과정에서 SOS4 균주는 *S. cerevisiae* 내 포도당 signal regulator의 역할을 하는 *MTH1* 유전자의 아미노산 염기서열에 돌연변이가 발생하였다.

본 연구에서는 해양 바이오매스에 다량 존재하는 포도당과 갈락토오스를 효율적으로 이용하도록 하여, 2,3-BD의 생

산에 있어 높은 수율과 생산성을 얻는 것이 최종 목표이다. 먼저, pyruvate decarboxylase 활성이 저해되고 이볼루션되어 *MTH1* 유전자에 돌연변이가 존재하는 균주 (SOS4)에 2,3-BD 생합성 경로를 도입한 균주 (BD4)의 회분식 배양을 통해 포도당과 갈락토오스의 동시 소모를 확인하였다. 따라서 이 결과가 *MTH1* 유전자의 돌연변이에 의한 효과인지를 확인하고자 이볼루션이 일어나지 않은 균주 (SOS2)와 인위적으로 *MTH1* 유전자에 돌연변이를 가하여 구축한 균주 (SOS2_Mth1)를 본 연구에 사용하였다. 이 두 균주에 2,3-BD 생합성 경로를 도입한 균주를 구축하였고, 회분식 배양 결과 *MTH1*에 돌연변이가 존재하는 균주 (BD2M)가 포도당과 갈락토오스를 동시에 소모함으로써 *MTH1* 와일드타입 균주 (BD2)에 비하여 *2*,3-BD의 생산성이 약 41% 증가한 수치를 보였다. 이 균주의 정확한 발효능력을 보기 위하여 fed-batch fermentation을 수행하였고, 마찬가지로 두 당을 동시 소모하여 약 75.5 g/L의 2,3-BD를 생산하였으나 발효 후반부로 갈수록 갈락토오스의 소모 속도가 느려지는

양상을 확인할 수 있었다.

두 번째로, 갈락토오스 소모 속도를 증진시키기 위해 갈락토오스 대사경로 내에 존재하는 5개의 유전자를 각각 과발현 시켜 균주를 구축하였다. 이들을 가지고 회분식 배양을 진행하였고, phosphoglucomutase를 암호화하는 *PGM2* 유전자를 과발현한 균주 (BD2M_PGM2)에서만 와일드타입 균주 (BD2M)에 비하여 약 13% 증가한 갈락토오스 소모 속도를 나타내었다. 이 균주의 정확한 발효능력을 보기 위하여 fed-batch fermentation을 수행하였고, 와일드타입 균주에 비해 약 42% 증가한 갈락토오스 소모 속도를 통해 약 39% 증가한 2,3-BD의 생산성을 확인할 수 있었다.

세 번째로, pyruvate decarboxylase 결여 효모의 단점인 탄소원에 의존적인 생장 체계를 극복하고자 선행연구자에 의해 확인된 *Candida tropicalis* 유래의 *pdc1* 유전자를 도입하고, 보효소 불균형으로 인해 부산물로 글리세롤이 다량 축적되는 문제점을 보완하고자 선행연구에 의해 확인된 *Lactococcus lactis* 유래의 *noxE* 유전자를 도입한

균주를 구축하였다. 이전 배양의 조건과는 달리 이 균주(BD2M_PGM2_Ctnox)의 배양시에는 외부 탄소원으로 에탄올을 공급하지 않고 진행하였다. 회분식 배양 결과, 와일드타입 균주 (BD2M_PGM2)에 비하여 약 19% 감소한 글리세롤의 수율을 확인할 수 있었으며, 탄소원에 비의존적인 생장이 가능해짐을 확인하였다. 이 균주의 정확한 발효능력을 보기 위하여 fed-batch fermentation을 수행하였고, 최종적으로 약 115.7 g/L의 2,3-BD를 생산하였으며 부산물인 글리세롤의 수율이 0.08 $g_{glycerol}/g_{sugars}$로 매우 낮은 수치를 보였다. 본 발효에서의 2,3-BD 생산 수율은 0.47 $g_{2,3\text{-}BD}/g_{sugars}$, 2,3-BD 생산성은 1.75 g/L/h를 나타내었다.

이를 통하여 재조합 *S. cerevisiae*인 BD2M_PGM2_Ctnox 균주는 해양 바이오매스로부터 2,3-BD를 효율적으로 생산할 수 있는 균주임을 증명하였다.

주요어 : 2,3-Butanediol, pyruvate decarboxylase, 갈락토오스, 해양 바이오매스, 혼합당 발효, mthl,

phosphoglucomutase, 포도당에 의한 전사억제, 보효소 불균형

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