A Thesis for the Degree of Master of Science

Improvement of 2,3-butanediol production from xylose in engineered *Saccharomyces cerevisiae*

재조합 효모를 이용한 자일로스로부터
2,3-butanediol 생산 증진

By

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Department of Agricultural Biotechnology
Seoul National University
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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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農 學 碩 士 學 位 論 文

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指導敎授 徐 鎭 浩

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ABSTRACT

2,3-Butanediol (2,3-BD) is a chemical compound with many industrial applications as a precursor of synthetic rubber and moistening and softening agents. Most of microbial production of 2,3-BD have been based on pathogenic bacteria, making difficult large-scale fermentations for 2,3-BD production. Therefore, GRAS (Generally Regarded As Safe) microorganisms including *Saccharomyces cerevisiae* would be desirable.

Production of 2,3-BD from cellulosic biomass is needed for sustainable development. Among various sugars, glucose and xylose are of special interest because they are abundant in cellulosic biomass. This thesis is aimed at production of 2,3-BD by using engineered *S. cerevisiae* capable of fermenting xylose.

To eliminate ethanol production, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* (SOS5) was constructed by deleting the *PDC* 1,

5 and 6 genes. The *XYL1*, *XYL2* and *XYL3* genes coding for xylose assimilating enzymes derived from *Scheffersomyces stipitis* were introduced into the SOS5 strain for xylose utilization. Then, the *alsS* gene encoding α-acetolactate synthase and the *alsD* gene encoding α-acetolactate decarboxylase both from *Bacillus subtilis* and endogenous *BDH1* gene were overexpressed (BD5X).

First, the 2,3-BD-producing Pdc-deficient BD5X and BD4X strains were compared for a performance of 2,3-BD production in a batch fermentation. The BD4X strain was constructed through deleting the *PDC1* and *PDC5* genes and had mutation on the *MTH1* gene, encoding a negative regulator of the glucose-sensing signal transduction pathway. BD5X produced 11.5 g/L of 2,3-BD from xylose with a yield of 0.34 $g_{2,3\text{-BD}}/g_{xylose}$. The titer of 2,3-BD increased 16% higher than the BD4X strain.

Second, to enhance xylose uptake rate in 2,3-BD production, the *TAL1* gene encoding transaldolase and the mutant XR gene encoding xylose reductase from *S.sitipitis* were expressed in the BD5X strain. The resulting strain, BD5X-TA1-mXR was tested for a performance of xylose consumption in batch fermentation under oxygen-limited conditions with BD5X as a control. BD5X-TA1-mXR produced 10.7 g/L of 2,3-BD from xylose with a productivity of 0.45 g/L/h. The productivity increased 66% higher in the BD5X-TAL1-mXR strain compare to the control strain BD5X strain. Also, the BD5X-TAL1 strain showed 1.85-fold higher specific activity of transaldolase than the control strain (0.24 versus 0.13 U/mg-protein) and 2.43-fold higher specific activity of xylose reductase than the control strain, BD5X-TAL1 (2.28 versus 0.42 U/mg-protein).

Finally, to solve defects about accumulation of glycerol and C_2-dependent growth in the Pdc-deficient strain, the *noxE* gene encoding

NADH oxidase from *Lactococcus lactis* and the *PDC1* gene encoding pyruvate decarboxylase 1 from *Candida tropicalis* were expressed in BD5X-TAL1-mXR. To test for a performance of 2,3-BD production, batch and fed-batch fermentations were carried out. With expression of *L. lactis noxE*, the yield of glycerol decreased by 40.1 % and the yield of 2,3-BD increased by 23.3% in BD5X-TAL1-mXR-nox compared with the control strain, BD5X-TAL1-mXR. With expression of *PDC* from *C. tropicalis*, xylose uptake rate increased by 33 % and productivity of 2,3-BD increased by 20.5 % in the BD5X-TAL1-mXR-nox-pdc compared with the BD5X-TAL1-mXR-nox strain.

Also, to test for C_2-independent growth, a batch fermentation was conducted without addition of ethanol. The BD5X-TAL1-mXR-nox-pdc strain was able to grow without addition of ethanol in a batch fermentation, as expected. In a fed-batch fermentation, the resulting strain produced 69.2 g/L of 2,3-BD from xylsoe with a low glycerol

yield, 0.08 $g_{glyerol}/g_{xylose}$, and the high yield of 2,3-BD of 0.38 $g_{2,3\text{-}BD}/g_{xylose}$. The titer of 2,3-BD produced from xylose in the BD5X-TAL1-mXR-nox-pdc strain was 58% higher than that obtained with the BD4X strain in a mixture of glucose and xylose under similar conditions.

These results suggested that the BD5X-TAL1-mXR-nox-pdc strain is suitable for producing 2,3-BD from cellulosic biomass.

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Keywords : 2,3-butanediol, pyruvate decarboxylase (Pdc)-deficient *S. cerivisiae*, Lignocellulose, xylose, transaldolase, mutant xylose reductase, redox balance, fed-batch, .

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I. INTRODUCTION

1. 2,3-Butanediol

2,3-Butanediol (2,3-BD) is a four-carbon compound and also known as 2,3-buylene glycol, dimethylethylene glycol or 2,3-dihydroxybutane. The chemical formula of 2,3-butanediol is $CH_3CH(OH)CH(OH)CH_3$ with molecular weight of 90.12 kDa. It has three stereoisomeric forms forms: levo-[D-(-)-,(*R, R*)-], dextro-[L-(+)-, (*S, S*)-] and meso-forms. (Figure. 1) (Syu et al, 2001; Celinska and Grajec et al, 2009).

2,3-BD is a promising platform chemical with extensive industrial applications since it can be converted into other valuable chemicals through dehydrogenation, ketalization, esterification and dehydration due to its low freezing point of -60℃.

2,3-BD can be converted to acetoin and diacetyl which is dehydrogenation products of 2,3-BD (Garg and Jain et al, 1995). 1,3-Butadien is the most promising application of 2,3-BD because it is a monomer for the production of synthetic rubber (van Haveren et al, 2008). In addition, 2,3-BD can be converted to methyl ethyl ketone (MEK) used as liquid fuel additives and polyurethane (Christensen et al, 2008; Garg and Jain et al, 1995).

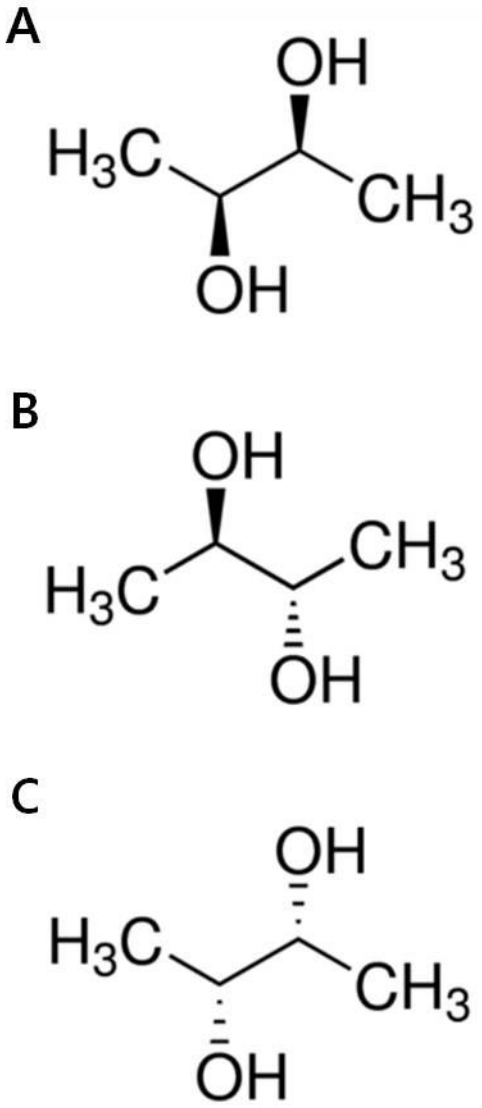

Figure 1. The stereoisomers of 2,3-butanediol.

(A) *L*-(+)-2,3-BD, (S,S)-Dextrorotatory form; (B) *meso*-2,3BD; (C) *D*-(-)-2,3BD, (R,R),-Levorotatory from.

2. 2,3-Butanediol production by bacteria

Production of 2,3-butanediol has been studied for a number of bacteria but only a few might can produce 2,3-BD (Garg et al, 1995). These bacteria are *Enterobacter aerogenes, Bacillus polymyxa, Klebsiella pneumoniae, K. oxytoca* and *Serratia marcescens*. Especially, *E. aerogenes* and *S. marcescens* are regarded as efficient microorganisms to produce 2,3-butanediol with high yield and productivity (Table. 1) (Celinska and Grajek et al, 2009).

In bacteria, 2,3-BD is produced along with by-products such as acetate, lactate, formate, succinate and ethanol via a mixed acid fermentation (Figure. 2) (Magee and Kosaric et al, 1987; Maddox et al, 1996). Four key enzymes are related in 2,3-BD production from pyruvate: α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), diacetyl reductase (DAR) and butanediol dehydrogenase (BDH) (Figure. 2) (Celinska and Grajek et al, 2009).

But, most bacteria are classified as pathogenic microbes (Class Ⅱ). Therefore the use of safe microorganisms is required in order to substitute for these pathogenic 2,3-butanediol producing bacteria (Kim and Timmusk et al, 2013 ;; Kim et al, 2013; Kim et al, 2015).

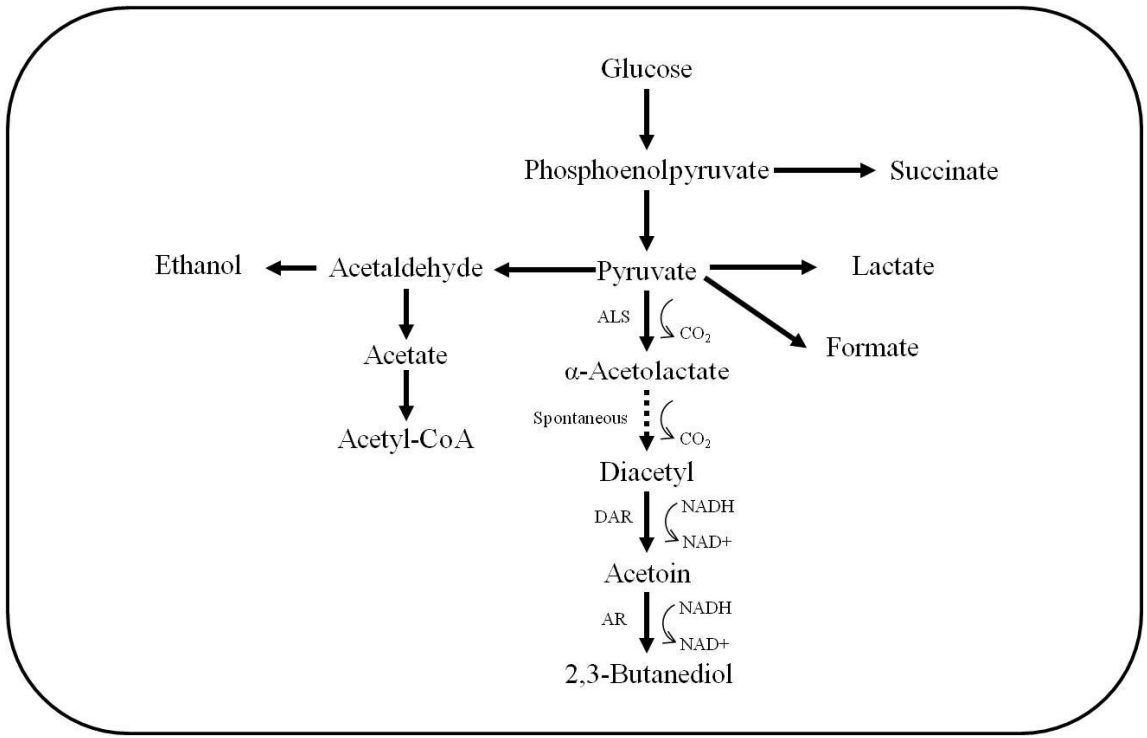

Figure 2. Mixed acid 2,3-butanediol pathway in bacteria. ALS : α-acetolactate synthase; DAR : diacetyl reductase; AR : acetoin reductase.

Table 1. Microbial 2,3-BD production

	Strain	Carbon source	Method	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
Bacteria	*Klebsiella pneumoniae*	Glucose, xylose and arabinose	Fed-batch	82.5	0.42	1.35	Wang, 2010
	Klebsiella oxytoca	Corncob hyrolysate	Fed-batch	35.7	0.50	0.59	Cheng, 2010
	K. oxytoca	Glucose	Fed-batch	13.0	0.47	1.62	Ji et al, 2010
	Enterobactor cloacae	Lignocellulosic hydrolysate	Fed-batch	119	0.47	2.30	Li, 2015
Yeast	*S. cerevisiae*	Glucose	Fed-batch	132	0.34	0.41	Choi, 2015
	S. cerevisiae	Glucose	Fed-batch	72.9	0.41	1.43	Kim & Hahn, 2015
	S. cerevisiae	Glucose, galactose	Fed-batch	100	0.35	0.31	Lian, 2014
	S. cerevisiae	**Xylose**	Batch	20.7	0.27	0.18	Kim, 2014

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

S. cerevisiae can synthesize a small amount of 2,3-BD naturally, however the yield and productivity of 2,3-BD are very low. (Garg and jain et al, 1995; Celinska and Grajek et al, 2009).

There are two pathways to produce 2,3-BD in *S. cerevisiae* (Fig. 3). Pyruvate is used to produce α-acetolactate by α-acetolactate synthase (*ILV2*) included the valine and isoleucine synthetic pathway. In *S. cerevisiae*, α-acetolactate decarboxylase does not exist unlike bacteria: acetoin cannot be converted into α-acetolactate enzymatically.

However, in the existence of oxygen, α-acetolactate can be converted into diacetyl by spontaneous decarboxylation. Diacetyl is conveted to acetoin by diacetyl reductase. Finally, acetoin is converted into 2,3-BD by butanediol dehydrogenase (*BDH1*) (Ehsani et al, 2009 ; Guymon and Crowell et al, 1967).

Recently, research on 2,3-BD production by engineered *S. cerevisiae* was done by using *in silico* genome scale metabolic analysis. Through deletion of *ADH1*, *ADH3* and *ADH5* genes coding for alcohol dehydrogenase, the engineered *S. cerevisiae* resulted in an improvement of 2,3-BD production by a 55-fold and reduced ethanol production compared to the wild type strain. The titer of 2,3-BD was

2.29 g/L and yield of 2,3-BD was 0.113 $g_{2,3\text{-BD}}$ / $g_{glucose}$ in the *S. cerevisiae* deficient in the *ADH* genes in anaerobic conditions (Ng *et al.* 2012).

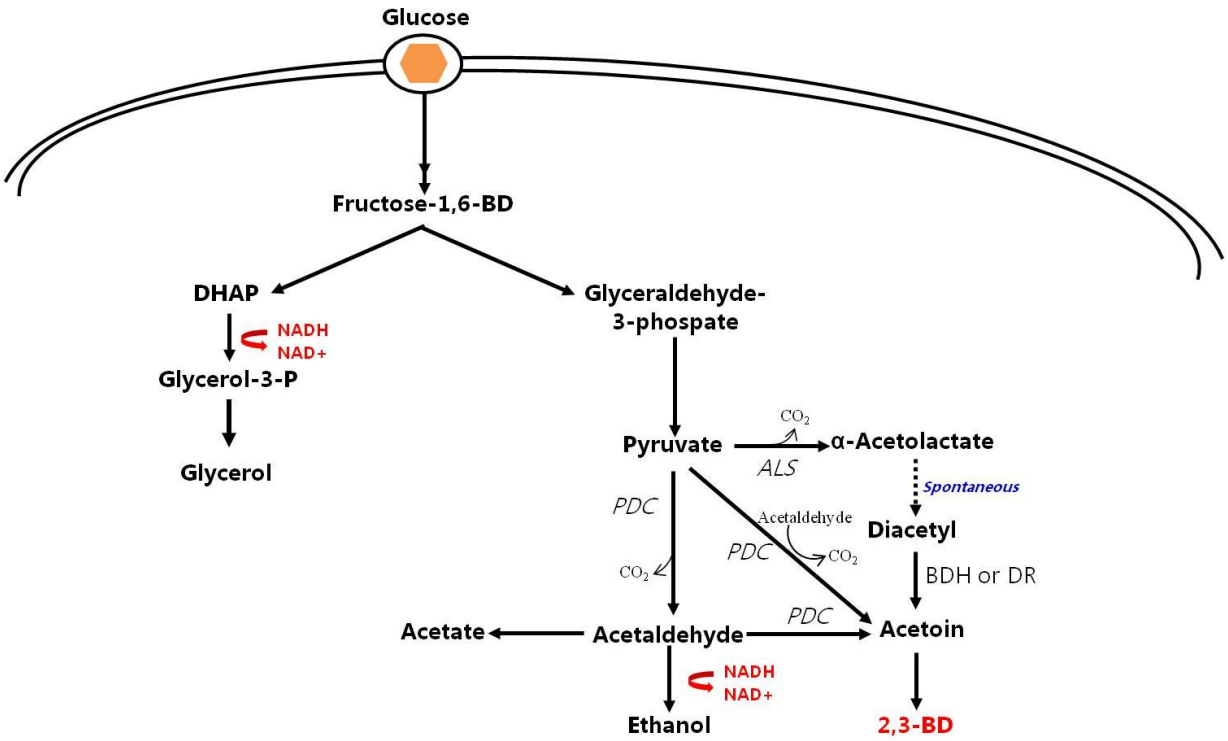

Figure 3. 2,3-BD biosynthesis pathway in *S. cerevisiae*. ALS : α-acetolactate synthase; PDC : pyruvate decarboxylase; BDH : 2,3-BD dehydrogenase

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

Traditionally, *S. cerevisiae* is used to ferment sugars to ethanol and 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 ethanol production toward the desired product. To maximize the production of desired products, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* has been selected as the host strain for production of lactate, malate and glycerol (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. Especially, pyruvate converts acetaldehyde to acetaldehyde, which is further reduced into ethanol by alcohol dehydrogenase. There are three structural genes (*PDC1, PDC5*, and *PDC6*) encoding pyruvate decarboxylase isoenzymes in *S. crevisiae*. Disruption of *PDC1* and *PDC5* or all *PDC* genes led to elimination of pyruvate decarboxylase activity completely (Flikweert et al, 1996).

The Pdc-deficient strains have potential defects for applications. First of all, the Pdc-deficient mutants have shown a two-carbon dependent cell growth pattern. In the other words, two-carbon compounds such as acetate or ethanol are necessary 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*. Without Pdc activity, acetaldehyde cannot be produced, which leads to the shortage of cytosolic acetyl-CoA. Secondly, the Pdc-deficient mutants showed lower growth rate on a glucose-containing medium than the wild type of *S. cerevisiae*. The Pdc-deficient strains suffer from oxidative stress because of redox imbalance due to the absence of ethanol synthesis. Therefore respiration is necessary for re-oxidation of cytosolic NADH in the Pdc-deficient strains

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. As a result, the point mutation of the *MTH1* gene in the SOS4 strains were likely to reduce

glucose uptake rate, which alleviates the pyruvate accumulation and redox imbalance in the evolved Pdc-deficient strains (Kim *et al.* 2013).

To produce 2,3-BD, the *alsS* and *alsD* genes coding for α–acetolactate synthase and α–acetolactate decarboxylase from *B. subtilis* were introduced to the engineered *S. cerevisiae* (SOS4) strain. Additionally, the endogeneous *BDH1* gene coding for 2,3-BD dehydrogenase was overexpressed in the SOS4 strain. 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 through fed-batch fermentation with a yield (0.28 $g_{2,3\text{-BD}} / g_{glucose}$) and a productivity (0.39 g 2,3-BD/L·h). However, substantial amounts of glycerol were also produced in parallel with 2,3-BD formation because of redox imbalance by excessive cytosolic NADH under oxygen-limited conditions (Kim et al, 2013).

There are a number of studies on the alteration of cytosolic concentration of NADH in *S. cerevisiae* through interconverting between NADH and NADPH, or 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 about expression of *L. lactis* NADH oxidase in the 2,3-BD-producing Pdc-deficient *S. cerevisiae*

reported a decrease in glycerol synthesis while increasing 2,3-BD production (Kim *et al.* 2015). Expression of the NADH oxidase result in decreasing intracellular NADH concentration and $NADH/NAD^+$ ratio and reducing glycerol accumulation in the Pdc-deficient *S. cerevisiae*.

Recently, it was reported about a solution of the defect of two-carbon compound dependent. The expression of the *PDC1* gene from *Candida tropicalis* with a fine-tuned expression level led to be growth without dependence of a two-carbon compound in Pdc-deficient *S. cerevisiae* (Kim *Ph.D. thesis.* 2016).

Also, in order to produce 2,3-BD from glucose and xylose, the *XYL1*, *XYL2*, and *XYL3* genes coding for xylose assimilating enzymes derived from *Scheffersomyces stipites* were introduced into the SOS4 strain. Additionally, the *alsS* and *alsD* genes from *B. subtilis* and endogenous *BDH1* gene were overexpressed to increase 2,3-butanediol production from xylose. The resulting strain (BD4X) produced 20.7 g/L of 2,3-butanediol from xylose with a yield (0.27 g 2,3-butanediol/g xylose) (Kim et al., 2014). Also, to utilize cellobiose, the celloextrin transpoter (*cdt-1*) and the β-glucosidase (*gh1-1*) from *Neurospora crassa* were expressed in the Pdc-deficient strain SOS2. Additionally,

the *alsS* and *alsD* gnens from *B. subtilis* were introduced. The resulting strain (SOS2-CB) produced 5.29 g/L of 2,3-butanediol from cellobiose with a yield (0.29 $g_{2,3\text{-BD}}$ / $g_{cellobiose}$) and a productivity (0.22 g 2.3-butanediol/L·h) (Nan et al., 2014). These results suggested the possibility of producing 2,3-butanediol sustainably and safely from cellulosic sugars.

5. Hemicellulose and xylose

Lignocellulose (Table 2) is the most abundant raw material on the earth including wood, crop and non-edible parts of food crops and fall into three main parts such as cellulose, hemicelluloses and lignin (Mosier et al, 2005). Hemicelluloses are the second abundant family of polymer on earth. Every year, the amount of unused hemicelluloses are 60 billion tons (Xu et al, 2006).

The structure of hemicelluloses is highly branched and complex heteropolymers such as hexose, pentose, and uronic acid. (Aristidou & Penttilä, 2000). The major part of hemocellulose is glucose and xylose. Glucose and xylose are most abundant sugars in cellulosic hydrolysate. In general, cellulosic hydrolysates consist of 60-70% glucose and 30-40% xylose (Mosier et al, 2005).

Therefore, rapid and efficient utilization of xylose is pre-requisite for production of bio-based chemicals from hemicellulosic biomass (Hahn-Hägerdal et al, 2006; Kim et al 2013).

Table 2. The polymer composition of lignocelluloses (IEA,2003)

Fraction	Content in lignocellulose	Major monomers
Cellulose	33-51%	Glucose
Hemicellulose	19-34%	Glucose, xylose, mannose, galactose and arabinose
Lignin	20-30%	Aromatic alcohols
Pectin	1-20%	Galacturnoic acid and rhamnose

6. Xylose metabolism in *S.cerevisiae*

Most researchs of 2,3-BD production from xylose have been performed for bacteria such as *K. pneumonia*, *K. oxytoca* and *E. aerogenes* (Cheng et al, 2010; Gao et al, 2010 ; Wang et al, 2010; Sun et al, 2009). But most of bacteria able to produce 2,3-BD are classified as potential pathogenic microorganisms no matter what ability to produce 2,3-BD efficiently. To produce 2,3-BD in an industrial-scale fermentation, a GRAS (Generally Recognized As Safe) organism is preferred for safety and industrialization.

S. cerevisiae is a very attractive organism because of numerous benefits in terms of safety and large-scale. But *S. cerevisiae* cannot metabolize xylose as a sole carbon source because it does not have inherent enzymes to metabolize xylose (Celinska and Grajek, 2009). To produce 2,3-BD from xylose in *S. cerevisiae*, the heterogous xylose-assimilating pathway has been introduced it (Ha et al, 2011; Karhumaa et al, 2007; Lee et al, 2012; Madhavan et al, 2009). The most general strategy to ferment xylose is introduction of the *XYL1* gene coding for xylose reductase (XR), *XYL2* gene coding for xylitol dehydrogenase (XDH) and *XYL3* gene coding for xylose kinase (XK) from *S. stipitis* to *S. cerevisiae*.

The study was performed to produce of 2,3-BD from xylose as a sole carbon source. But productivity and concentration of 2,3-BD were still low compare to bacteria (Figure 4) (Kim et al, 2013b ; Ji et al, 2011)

Several studies have been reported to improve xylose utilization in *S. cerevisiae* through modulation of genes related in the pentose phosphate pathway in *S. cerevisiae* (Matsushika et al, 2012; Johansson and Hahn-Hägerdal et al, 2002; Walfridsson et al, 1995). Over expression of the *TAL1* gene, encoding transaldolase in the pentose phosphate pathway, has been reported to increase the growth rate and productivity (Jin et al, 2005)

Also, there have been studies about the XR gene encoding xylose reductase. XR coded by *XYL1* gene catalyzes the step of xylose to xylitol using NADPH or NADH as a cofactor. Most of XRs use NADPH as a cofactor, but recently, the NADH-preferring XR mutant was constructed by protein engineering (Jo et al, 2015; Lee et al, 2012; Watanabe et al, 2007). In Pdc-deficient *S. cerevisiae*, NADH accumulated in the cytosol under oxygen-limited conditions (Kim et al, 2013b). Therefore, the NADH-preferring XR mutant is desirable to improve 2,3-BD productivity from xylose in Pdc-deficient *S. cerevisiae*.

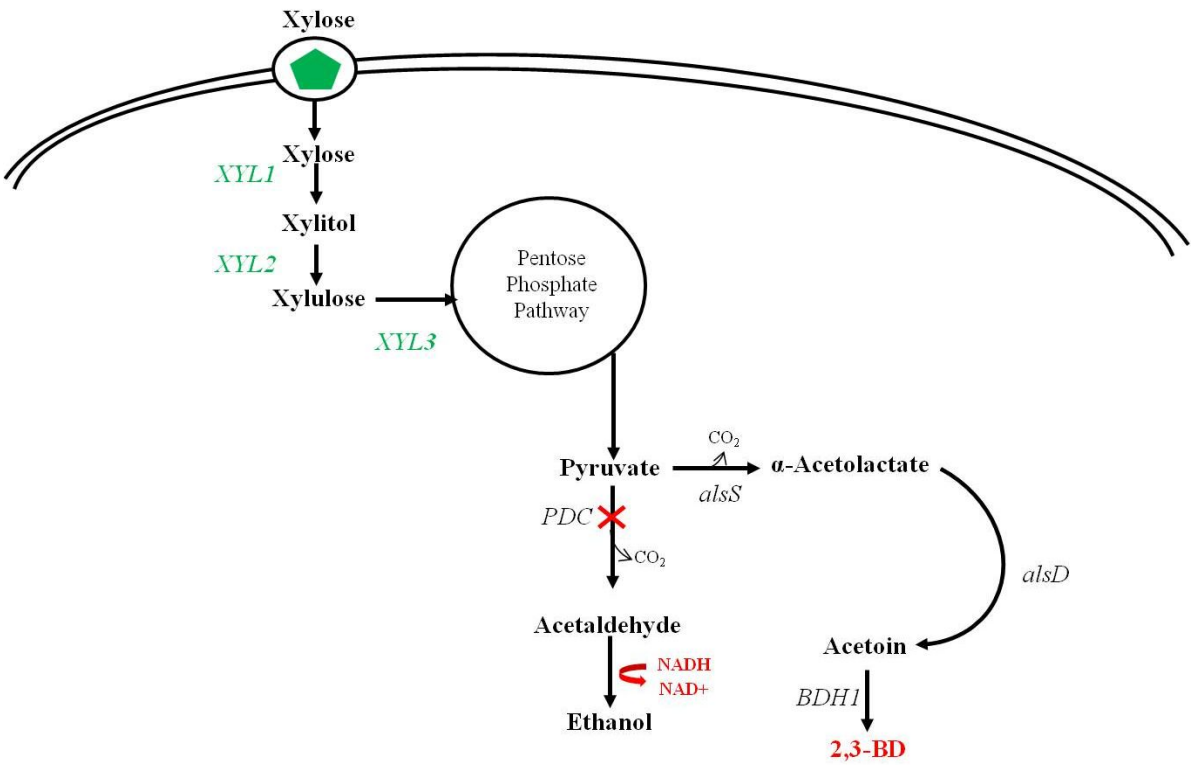

Figure 4. 2,3-BD pathway from xylose in *S. cerevisiae*. *XYL1* : xylose reductase from *Scheffersomyces sitpitis*; *XYL2* : xylitol dehydratase from *S. sitpitis*; *XYL3* : xylulose kinase from *S. sitpitis*; *alsS* : α-acetolactate synthase from *Bacillus subtilis*; *alsD* : α-acetolactate decarboxylase from *B. subtilis*

7. Research objectives

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

1) To select a pyruvate decarboxylase deficient strain as a 2,3-BD producing host strain

2) To enhance the xylose metabolic pathway via co-expressing the genes for TAL and mutant XR.

3) To maximize the fermentation performance of the BD5X-TAL1-mXR-nox-pdc strain by optimizing a fed-batch fermentation process.

Ⅱ. Materials and Methods

1. Reagents

All chemicals used were of reagent grade. Glucose, xylitol, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), β-nicotinamide adenine dinucleotide sodium salt, β-nicotinamide adenine dinucleotide 2′-phosphate, D-Fructose 6-phosphate disodium salt hydrate, D-erythrose 4-phosphate sodium salt, α-glycerophosphate dehydrogenase-triosephosphate isomerase from rabbit muscle were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Bacto-peptone, bacto-trypton, yeast extract and bacto-agar were obtained from Difco (Detroit, MI., USA); xylose from Junsei (Tokyo, Japan); glycylglycine free base and ethanol from Merck (Darmstadt, Germany); NaCl, NaOH, sodium phosphate, HCl, H_2SO_4, magnesium chloride hexahydrate, potassium phosphate monobasic and potassium phosphate dibasic from Duksan (Ansan, Korea).

2. Strains and plasmids

2.1. Strains

To propagate and preparate plasmid DNA, *E. coli* TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used.

S. cerevisiae D452-2 [*Matα, leu2 his3 ura3 can1*] and the Pdc-deficient *S. cerevisiae* D452-2 strain (SOS5) was used as host strains for the expression of the genes involved in the 2,3-butanediol metabolic pathway. (Table 3)

The BD4X were constructed by Dr. S. J. Kim at Seoul National University in Korea (Kim et al. 2013). The SOS5 and BD5 were constructed by Dr. J. W. Kim at Seoul National University in Korea (Kim et al. 2015). Other strains in Table 2 were constructed in this study.

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

Table 3. List of the strains used in this study

Name	Description	Reference
D452-2	*S. cerevisiae* Matα leu2 his3 ura3 can1	Kim, 2013a
SOS4	D452-2 ΔPDC1, ΔPDC5 (evolved)	Kim, 2013a
BD4X	SOS4 pSR6-XYL123, p426GPD_alsS_alsD, p425GPD_BDH1	Kim, 2014
SOS5	D452-2 ΔPDC1, ΔPDC5, ΔPDC6	Kim, 2015
BD5	SOS5 p426GPD_alsS_alsD, p425GPD_BDH1	Kim, 2015
SOS5X	SOS5 pRS306-X123, p426GPD_alsS_alsD, p425GPD_BDH1	In this study
BD5X	SOS5 pRS306-X123, p426GPD_alsS_alsD, p425GPD_BDH1	In this study
BD5X-TAL1	BD5X p423GPD_alsS_alsD, p425GPD_BDH1_TAL1	In this study
BD5X-TAL1-mXR	BD5X-TAL1 pITy-3_XR^{mut}	In this study
BD5X-TAL1-mXR-nox-pdc	BD5X-TAL1-mXR p414AUR_noxE_PDC1	In this study

2.2. Plasmids

Three plasmids were used as mother vectors which have the *GPD 7* promoter and *CYC1* terminator from *S. cerevisiae.* (Figure. 5) These are cloning vectors for an episomal expression system of the 2,3-butanediol biosynthetic pathway, *alsS* and *alsD* genes from *B. subtilis*, *BDH1* genes from *S. cerevisiae* and *TAL1* gene from *S. stipites*.

In order to construct a xylose-fermenting Pdc-deficient strain (SOS5X), XcmI-treated pRS306_XYL123 harboring *XYL1*, *XYL2* and *XYL3*, was integrated into the URA3 locus of the SOS5 genome.

And 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*. Also, δ-integration of plasmid pITy-3_XR^{mut} was used. (Table 4).

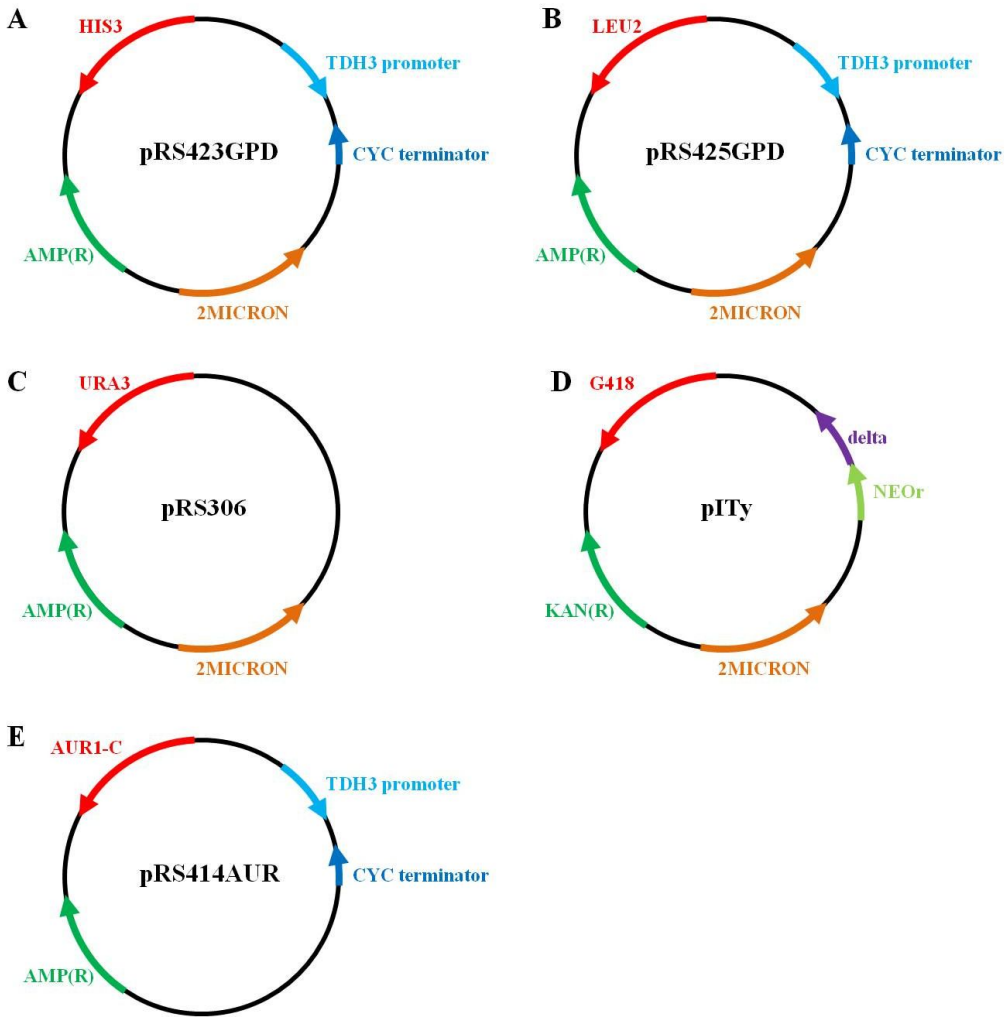

Figure 3. Mother vectors used in this study.

(A) p423GPD, (B) p425GPD, (C) pRS306, (D) pITy, (E) p414AUR

Table 4. List of the plasmids used in this study

Name	Description	Reference
pRS423	*HIS3*, *TDH3* promoter, *CYC* terminator, 2μ origin, Amp^r	Christianson et al, 1992
pRS425	*LEU2*,	Christianson et al, 1992
pRS306	*URA3*, 2μ origin, Amp^r	Sikorski and Hieter et al, 1989
pITy	2μ origin, $G418^r$, KAN^r	de Albuquerque et al, 2014
pRS414AUR	AUR1-C, *TDH3* promoter, *CYC* terminator, 2μ origin, Amp^r	Kim, theis, 2016
pRS306-X123	pRS306 $TDH3_p$-XYL1-$TDH3_t$, $PGK1_p$-XYL2-$PGL1_t$, $TDH3_p$-XYL3-$TDH3_t$ *XYL1*, *XYL2*, *XYL3* gene from *Scheffersomyces stipitis*	Kim et al, 2012
p423TDH3_alsS_alsD	pRS423 $TDH3_p$-alsS-CYC_t, $TDH3_p$-alsD-CYC_t *alsS*, *alsD* gene from *Bacillus subtilis*	Kim et al, 2014
p425TDH3_BDH1	pRS425 $TDH3_p$-BDH1-CYC_t *BDH1* gene from *S. cerevisiae* D452-2	Kim et al, 2013
p425TDH3_TAL1	pRS425 $TDH3_p$-TAL1-CYC_t *TAL1* gene from *S. stipitis*	In this study
p426TDH3_BDH1_TAL1	pRS425 $TDH3_p$-BDH1-CYC_t, $TDH3_p$-TAL1-CYC_t *TAL1* gene from *S. stipitis*	In this study

pITy-3_XR^{mut}	pITy-3XR^{mut} (R276H) *XR* gene from *S. stipitis*	Jo et al, 2015
p414AUR_noxE	pRS414AUR TDH3$_p$-noxE-CYC$_t$ *noxE* gene from *Lactococcus lactis*	Kim thesis, 2016
p414AUR_noxE_PDC1	pRS414AUR TDH3$_p$-noxE-CYC$_t$, GPD2$_p$-PDC1-CYC$_t$ *PDC1* gene from *Candida tropicalis*	Choi thesis, 2016

Table 5. List of oligonucleotide used in this study

Primer name	Oligonucleotide sequence (5'→3')
F_TDHp_seq	GTAGGTATTGATTGTAATTCTGTAAAT
R_CYCt_seq	ATAACTATAAAAAAATAAATAGGGAC
F_Sma1_psTAL1	TCCCCCGGGGGAATGTCCTCCAACTCCCTTG
R_Xho1_psTAL1	CCGCTCGAGTTATTAATAGAAGCGAAATATGTAAATACC
F_Pst1_TDH3p	AAAACTGCAGAGTTTATCATTATCAATACTCGCCATTTC
R_Nde1_CYCt	GGAATTCCATATGGGCCGCAAATTAAAGCCTTC
R_HindⅢ_psTAL1	CGGGATCCAAAATGAGAGCTTTGGCATATTTC
R_mid_psTAL1_seq	GATGGCTCTGACAGAAATCACAC

3. DNA manipulation and transformation

3.1. Enzymes

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

3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed with the Accupower™ PCR PreMix (Bioneer Co., Daejon, Korea) in 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* ™ 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 genes was carried out using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany) according to the manufacturer's instruction.

3.4. Transformation of *E. coli*

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

for 45 sec, and 1 mL of LB medium was added to the test tubes and incubated at 37℃ for 1 hour to allow the bacteria to express the antibiotic resistance. Transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.5. Yeast transformation

Transformation of expression vectors was performed using the yeast EZ-Transformation kit (BIO 101, Vista, Calif.). Transformants were selected on YNB medium containing 20 g/L glucose with 1.5 g/L ethanol. Amino acids and nucleotides were added as necessary.

4. Media and culture conditions

4.1. Media

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

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

4.2. Batch fermentations

Batch fermentation was performed in a 250 mL flask with 50 mL working volume of YP medium containing 40 g/L xylose with 1.5 g/L ethanol at 30 °C in a shaking incubator (Vision Korea), and shaking rate was maintained at 80 rpm for creating micro-aerobic conditions.

Seed cultures were prepared by culturing in a 5 mL test tube of YNB medium containing 20 g/L glucose with 1.5 g/L of ethanol and

incubated overnight at 30 ℃, 250 rpm in a shaking incubator (Vision, Korea). Pre-cultures were prepared by inoculating the seed cultures in a 500 mL baffled flask with 200 mL working volume of YNB medium containing 20 g/L glucose with 1.5 g/L ethanol and grown in a shaking incubator at 30°C and 220 rpm for 72 h.

Yeast cells were harvested at a mid-exponential phase and inoculated into main cultures with initial OD_{600} of ~10.

4.3. Fed-batch fermentations

To obtain a high concentration of 2,3-butanediol, fed-batch fermentation was carried out using a 1Lbench-top fermenter (FERMENTEC, Korea). Fed-batch fermentation was performed in 500 mL of YP medium with 80 g/L xylose at 30℃ and pH 5.5. 200 rpm of agitation speed and 0.5 vvm of aeration were maintained throughout the cultivation.

Seed cultures were prepared by culturing in a 5 mL test tube of YNB medium containing 20 g/L glucose with 1.5 g/L ethanol in a shaking incubator at 30°C and 250 rpm for 72 h. Pre-cultures were prepared by inoculating the seed cultures in a 500 mL baffled flask

with 100 mL working volume of YNB medium containing 20 g/L glucose and grown in a shaking incubator at 30°C and 220 rpm for 72 h.

Yeast cells were prepared by growing cells overnight to an OD_{600} of 10. The cells were harvested by centrifugation at 9000 rpm for 5 min and washed in 5 mL of sterilized DDW and inoculated into the bioreactor with initial OD_{600} of 10. After depletion of xylose, additional xylose solutions were added.

5. Analysis

5.1. Dry cell weight

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

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

5.2. Metabolite detection.

Concentrations of glucose, xylose, 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°C and eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min.

5.3. Protein concentration

Protein concentration was determined by the protein assay kit (Bio-Rad, Richmond, CA, USA) based on the method of Bradford using bovine serum albumin (BSA) as standard. Combined samples were incubated at 30°C and measured absorbance at 595 nm.

6. Measurement of enzyme activities

6.1. Sample preparation

To prepare crude extracts, about 1 x 10^9 mid-exponential phase cells grown on the YNB medium with 40 g/L glucose and 1.5 g/L ethanol in a flask culture were harvested and washed twice with DDW. The protease inhibitor (Roche, Switzerland) was added and the harvested cells were lysed with Yeast Protein Extraction Reagent (Thermo Scientific, MA). After centrifugation for 20 min at 12,000 rpm and 4°C, the supernatants were used to determine the transaldolase and xylose reductase activity within 3 hours and diluted with DDW as necessary.

6.2. Transaldolase activity assay

The transaldolase (TAL) activity assays were performed at 30°C with the reaction mixture 67 mM glycylglycine buffer (pH 7.7), 2 mM D-erythrose 4-phosphate, 6.7 mM D-fructose 6-phosphate, 15 mM magnesium chloride, 0.13 mM ß-NADH, 0.01 mg of α-glycerophophate dehydrogenase/triosephoshate isomerase with some modifications based on a previous report (Bergmeyer et al, 1974).

The reactions were initiated by adding a crude extract, and a decrease of absorbance at 340 nm was measured. One unit of activity was defined as 1.0 μmole of D-glyceraldehyde 3-phosphate from D-fructose 6-phosphate per minute in the presence of D-erythrose 4-phosphate, at pH 7.7 at 30°C in a coupled system with a-GDH/TPI and ß-NADH. Specific enzyme activity (U/mg protein) was estimated by dividing enzyme activity by the cellular protein concentration.

6.2. Xylose reductase activity assay

The xylose reductase (XR) activity assays were based on a previous report with some modifications (Jeun et al, 2003). XR activity was determined by measuring the oxidation of NADPH or NADH at 30°C. One unit of xylose reductase activity was defined as the amount of an enzyme that can oxidize 1.0 μmole of NADPH or NADH per minute. Specific enzyme activity (U/mg protein) was estimated by dividing enzyme activity by the cellular protein concentration.

III. RESULTS AND DISCUSSIONS

1. Selection of 2,3-BD producing Pdc-deficient *S. cerevisiae*

In the previous study by Dr. S.J. Kim, introducing the *alsS* and *alsD* genes from *B. subtilis* and overexpressing the endogenous *BDH1* gene and the *XYL1*, *XYL2* and *XYL3* genes from *S. stipites* in pyruvate decarboxylase-deficient *S. cerevisiae* (SOS4) were performed. The resulting strain, BD4X, reported the productivity of 2,3-BD as 0.18 g/L/h (Kim et al, 2014). It was relatively low compared to glucose based production of 2,3-BD in engineered *S. cerevisiae* because of the mutation of the *MTH1* gene which encodes a negative regulator of the glucose signaling pathway (Kim et al, 2013; Oud et al, 2012; van Maris et al, 2004).

Therefore, in the follow-up study by Dr. J.W. Kim, SOS5 strain by deletion the *PDC 1, 5, 6* genes without evolution and then introducing the *alsS* and *alsD* genes from *B. subtilis* and overexpressing the endogenous *BDH1* gene were performed. The resulting strain BD5 showed a two-carbon dependent cell growth pattern (Kim et al, 2015).

In this study, to utilize xylose as a sole carbon source for production of 2,3-BD, BD5X were constructed from BD5 strain via introducing of the *XYL1*, *XYL2* and *XYL3* genes.

To compare between BD4X and BD5X, flask batch cultivation of both BD4X and BD5X were carried out using 40 g/L xylose (Figure 6).

While BD4X produced 9.34 g/L of 2,3-BD, BD5X produced 11.5 g/L of 2,3-BD from xylose within 48 h under micro-aerobic condition. Also, 2,3-BD productivity were higher in BD5X(0.24 g/L/h) than BD4X (0.19 g/L/h) (Table 6).

Because mutation of *MTH1* in BD4X strain led to repress hxt transcription, BD4X transported xylose more slowly than BD5X. To increase 2,3-BD productivity, BD5X strain was used to next studies.

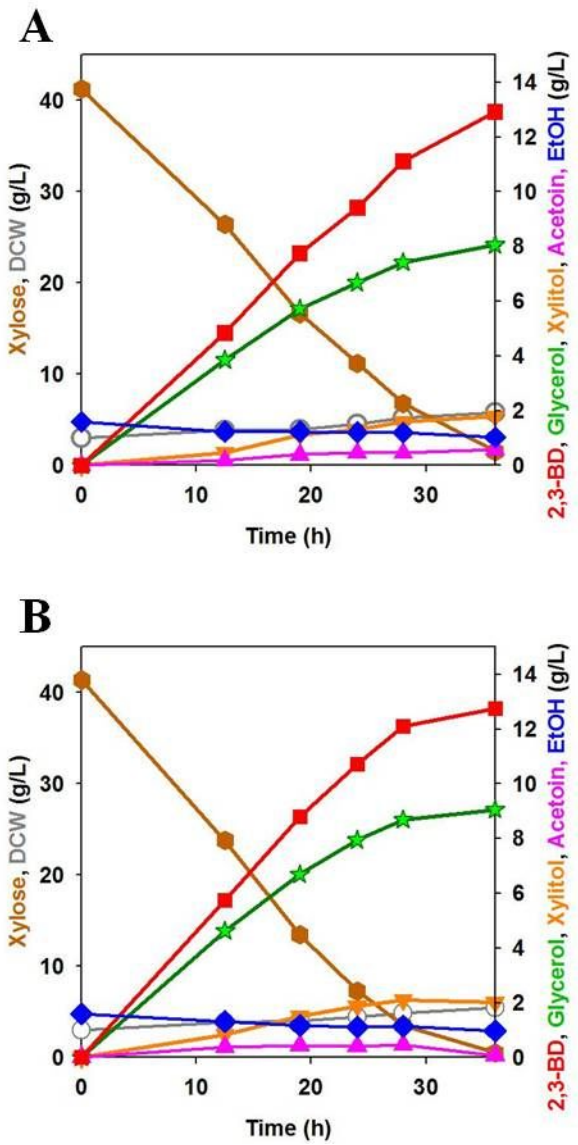

Figure 6. Batch fermentation of (A) BD4X, (B) BD5X

Symbols : Xylose ( ), DCW ( ), Xylitol ( ), Glycerol ( ), Acetoin ( ), 2,3-BD ( ), Ethanol ( )

Table 6. Summary of batch fermentation in part 1

Strains	Xylose uptake rate (g/L/h)	Xylitol (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD4X	0.65	1.39	3.10	0.34	9.34	0.30	0.19
BD5X	0.70	1.38	5.90	0.40	11.5	0.34	0.24

* All value calculated at 48h

2. Enhancement of xylose metabolic pathway to increase xylose uptake rate in 2,3-BD production

2.1 Expression of *TAL1* gene

To enhance the xylose assimilation pathway, *S. stipites TAL1* were introduced in BD5X strain using plasmid under the *TDH3* promoter. The resulting strain, BD5X-TAL1 was measured by NADH assay. BD5X strain was used as control. As shown in Figure 7, BD5X-TAL1 strain exhibited transaldolase activity that was 1.85 times higher than control strain (0.24 versus 0.13 U/mg-protein).

Batch fermentation was done in YP medium with 40 g/L xylose with 1.5 g/L of ethanol under micro-aerobic conditions. Results of batch fermentation were in Figure 8. Table 7 summarizes the xylose consumption and product yields for both strains at 28 h. The BD5X-TAL1 strain consumed more than 48.2 % as much xylose as the BD5X strain with 40 g/L of xylose. And the productivity of 2,3-BD was increased 48.1% in BD5X-TAL1 strain compared BD5X strain. Based on this data, BD5X-TAL1 strain was used for 2,3-butanediol production.

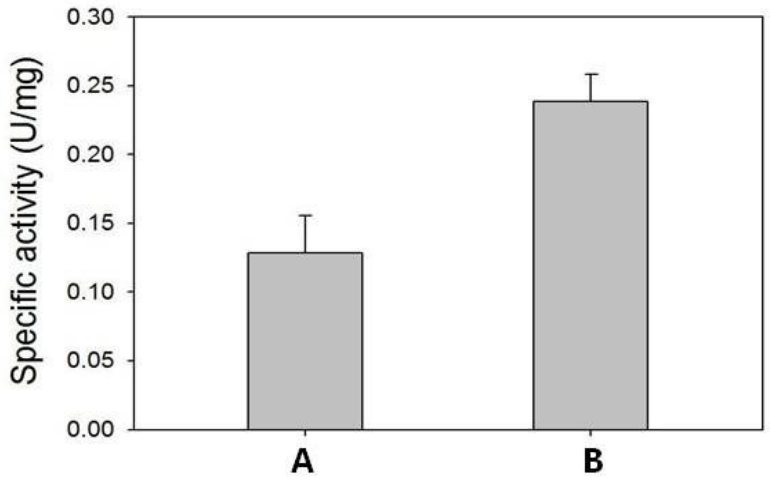

Figure 7. Comparison of the specific activity of transaldolase (TAL1)

(A) BD5X, (B) BD5X-TAL1

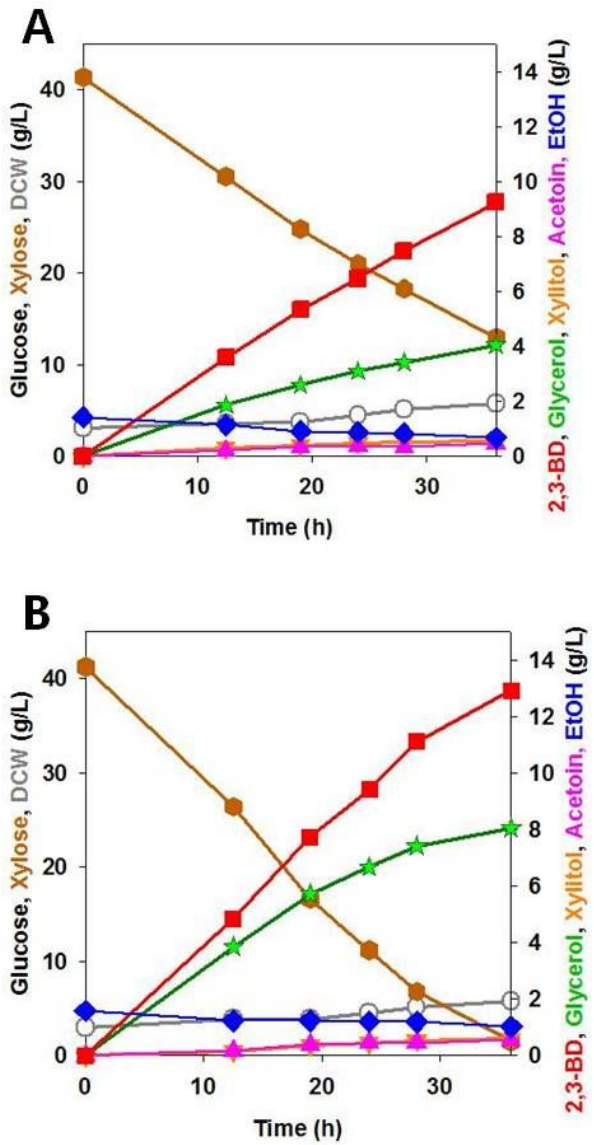

Figure 8. Batch fermentation of (A) BD5X, (B) BD5X-TAL1

Symbols : Xylose ( ), DCW ( ), Xylitol ( ), Glycerol ( ), Acetoin ( ), 2,3-BD ( ), Ethanol ( )

Table 7. Summary of batch fermentation in part 2.1

Strains	Xylose uptake rate (g/L/h)	Xylitol (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD5X	0.83	1.59	3.43	0.36	7.47	0.32	0.27
BD5X-TAL1	1.23	1.60	7.41	0.45	11.1	0.32	0.40

* All value calculated at 28h

2.2 Expression of mutant *XR* gene

It was reported that the *XR* mutant has property of NADH-preferring xylose reductase activity (Jo et al, 2015; Lee et al, 2012; Watanabe et al, 2007). To re-oxidate of cytosolic NADH in the Pdc-deficient BD5X-TAL1 strain, *XYL1* mutant was introduced by delta-integration plasmid. The resulting strain, BD5X-TAL1-mXR was measured to confirm the effect of XR mutant by NADH assay. BD5X-TAL1 was used as a control strain. As shown in Figure 9, BD5X-TAL1-mXR strain exhibited NADH-specific activity that was 5.43 times higher than control strain (2.28 versus 0.42 U/mg-protein).

Batch fermentation was done in YP medium with 40 g/L xylose and 1.5 g/L of ethanol under micro-aerobic conditions. The results of batch fermentation were shown Figure 10. The BD5X-TAL1 strain consumed more than 13.6 % as much xylose as the BD5X strain. And the productivity of 2,3-BD was increased 15.4 % in BD5X-TAL1 strain compared to BD5X strain.

Based on this data, BD5X-TAL1-mXR strain was used for 2,3-butanediol production.

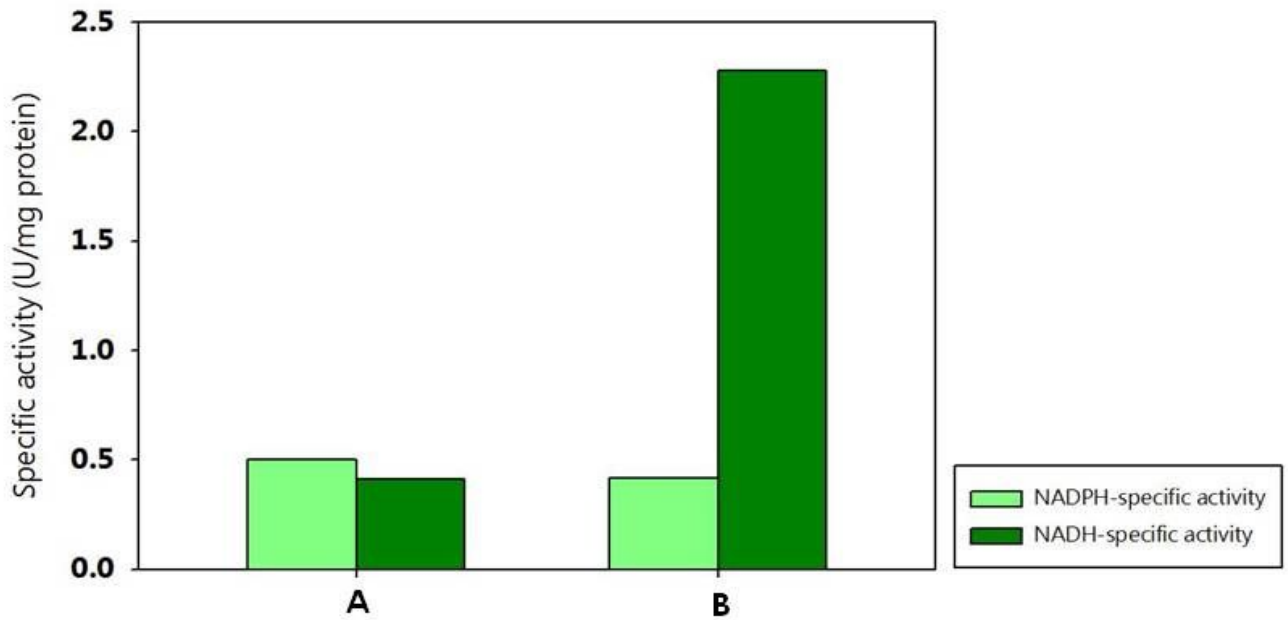

Figure 9. Comparison of the specific activity of transaldolase (TAL1)

(A) BD5X-TAL1 (B) BD5X-TAL1-mXR

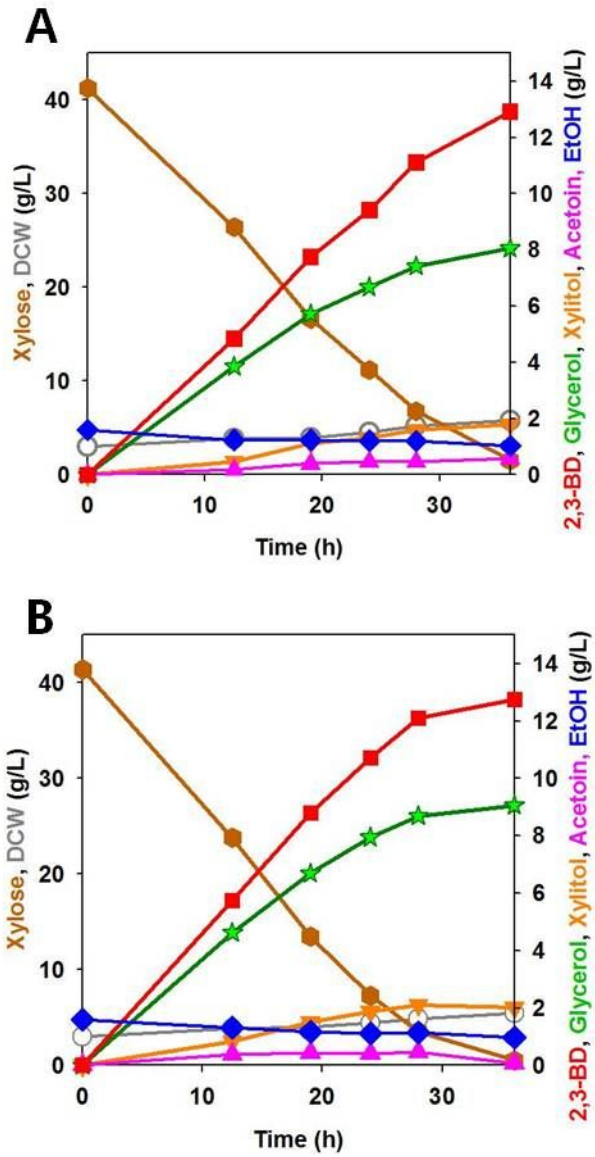

Figure 10. Batch fermentation of (A) BD5X-TAL1, (B) BD5X-TAL1-mXR

Symbols : Xylose ( ), DCW ( ), Xylitol ( ), Glycerol ( ), Acetoin ( ), 2,3-BD ( ), Ethanol ( )

Table 8. Summary of batch fermentation in part 2.2

Strains	Xylose uptake rate (g/L/h)	Xylitol (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD5X-TAL1	1.25	1.31	6.67	0.46	9.42	0.31	0.39
BD5X-TAL1-mXR	1.42	1.88	7.94	0.4	10.7	0.31	0.45

* All value calculated at 48h

3. Expression of *L. lactis noxE* and *C. tropicalis PDC1*

There are two kinds of potential defect in the Pdc-deficient strain. First, glycerol was produced in parallel with 2,3-BD formation because of redox imbalance in the cytosol. In the previous study, *noxE* from *L. lactis* was introduced in Pdc-deficient *S. cerevisiae*. As a result, yield of glycerol was 65.3% decrease and yield of 2,3-BD was 23.8% increase on glucose-based fermentation (Kim et al, 2015).

Second of the potential defects is a need for external supplement of a 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, *C. tropicalis PDC1* was introduced at a low expression level in the Pdc-deficient strain. As a result, two-carbon independent cell growth was observed (Kim *thesis*, 2016).

In this study, to decrease glycerol accumulation and to ferment without a two-carbon supplementation such as ethanol, *noxE* and *PDC1* were introduced in BD5X-TAL1-mXR strain using p414AUR_noxE and p414_AUR_noxE_PDC1 plasmid.

To confirm the effect of NADH oxidase, batch fermentation were carried in YP medium with 40 g/L xylose and 1.5 g/L of ethanol under micro-aerobic conditions. BD5X-TAL1-mXR was used as a

control strain. The results of batch fermentation were shown Figure 11. With expression the *noxE* gene, yield of glycerol was 40.1 % decrease and yield of 2,3-BD was 23.3% increase compared with control strain. Based on this data, BD5X-TAL1-mXR-nox strain was used for 2,3-butanediol production.

And the resulting strain, BD5X-TAL1-mXR-nox-pdc with p414AUR_noxE_PDC1 plasmid was tested. Batch fermentation were carried in YP medium with 40 g/L of xylose and 1.5 g/L of ethanol under micro-aerobic conditions. The results of batch fermentation were shown Figure 11. With expression *C. tropicalis*, xylose uptake rate was 33 % increase and productivity of 2,3-BD was 20.5 % increase compared with BD5X-TAL1-mXR-nox strain.

To evaluate the fermentation ability of the BD5X-TAL1-mXR-nox-pdc strain on xylose, fed-batch fermentation was carried out without ethanol (Figure 12). The initial fermentation medium contained 80 g/L of xylose. In consideration of the characteristics of the NADH oxidase encoded by *noxE* gene, agitation was changed during the cultivation: from 300 rpm to 200 rpm to reduce accumulation of acetoin according to the method of the previous research (Kim *thesis* et al, 2016).

The fed-batch fermentation resulted in 69.2 g/L of 2,3-BD during 66 hours of cultivation with a yield of 0.38 $g_{2,3\text{-BD}}$ / g_{xylose} and a productivity of 0.28 g/L/h (Table 9). These are the higher titer of 2,3-BD and lower yield of glycerol from the fed-batch fermentation than those from batch fermentation. The titer of 2,3-BD produced from xylose in BD5X-TAL1-mXR-nox-pdc strain was 58% higher than from glucose and xylose mixture in BD4X strain under a fed-batch fermentation.

The introduction of *L. lactis noxE* and *C. tropicalis PDC1* could be a strategy for Pdc-deficient *S. cerevisiae* to produce bio-based chemicals. In addition, the resulting strain BD5X-TAL1-mXR-nox-pdc strain could be a desirable strain for producing 2,3-BD from cellulosic biomass for applications.

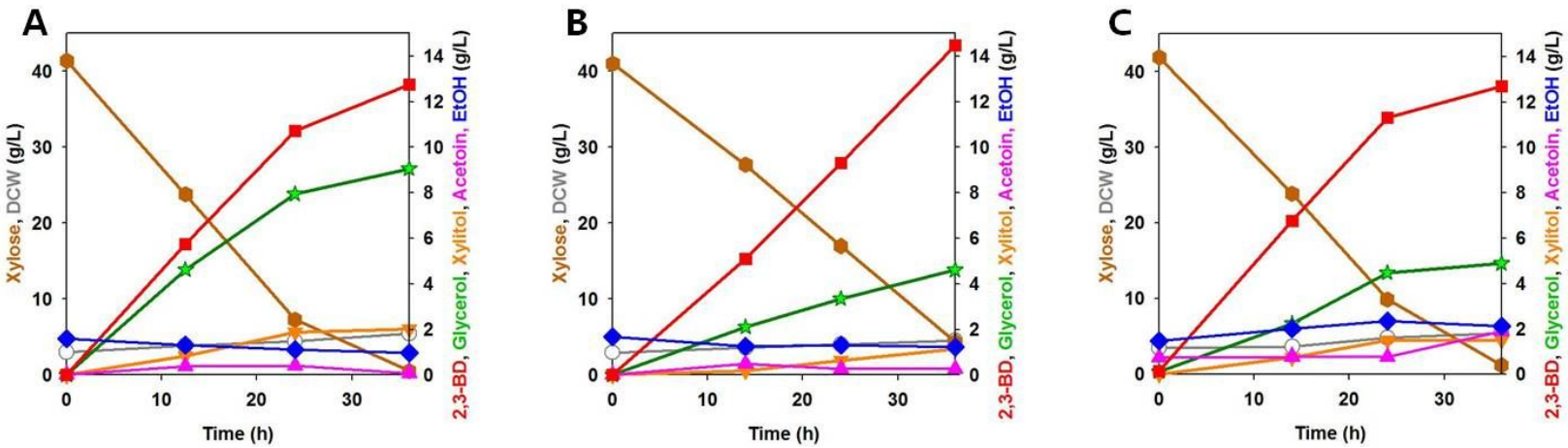

Figure 11. Batch fermentation of (A) BD5X-TAL-mXR, (B) BD5X-TAL-mXR-nox, (C) BD5X-TAL1-mXR-nox-pdc

Symbols : Xylose ( ), DCW ( ), Xylitol ( ), Glycerol ( ),

Acetoin ( ), 2,3-BD ( ), Ethanol ( )

Table 9. Summary of batch fermentation in part 3

Strains	Xylose uptake rate (g/L/h)	Xylitol (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD5X-TAL1-mXR	1.42	1.88	7.94	0.4	10.7	0.23	0.31	0.45
BD5X-TAL1-mXR -nox	1.00	0.63	3.35	0.25	9.31	0.14	0.39	0.39
BD5X-TAL1-mXR-nox-pdc	1.33	1.5	4.48	0.77	11.3	0.12	0.35	0.47

* All value calculated at 24h

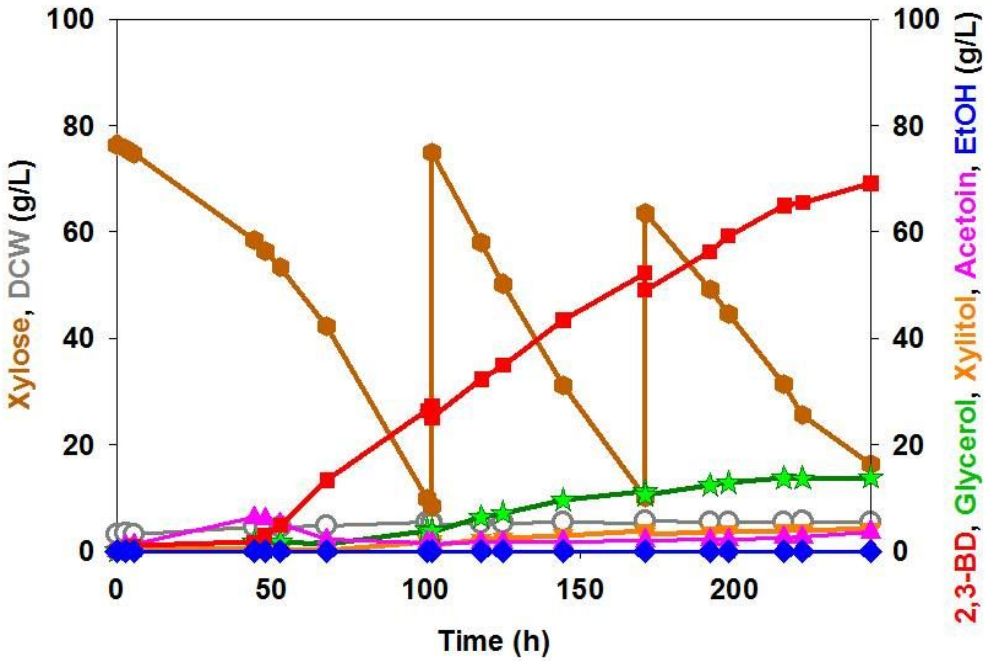

Figure 12. Fed-batch fermentation of BD5X-TAL-mXR-nox-pdc

Symbols : Xylose ( ), DCW ( ), Xylitol ( ), Glycerol ( ), Acetoin ( ), 2,3-BD ( ), Ethanol ( )

Table 10. Summary of fed-batch fermentations

Strains	Xylose uptake rate (g/L/h)	Xylitol (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD5X-TAL1-mXR -nox-pdc	0.74	0.42	13.8	3.63	69.2	0.08	0.38	0.28

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) The Pdc-deficient *S. cerevisiae*, BD5X strain was selected as a 2,3-BD producing host strain in terms of titer and productivity of 2,3-BD.

(2) To enhance xylose utilization in 2,3-BD producing *S. cerevisiae*, the *TAL1* and *XR* genes from *S. stipitis* were expressed in BD5X, a pdc-deficient strain. The resulting strain BD5X-TAL1-mXR was able to produce 11.1 g/L of 2,3-BD and 0.45 g/L/h of 2,3-BD productivity.

(3) To decrease the production of glycerol, a main byproduct and to solve the C_2-dependent growth of 2,3-BD-producing Pdc-deficient *S. cerevisiae* on xylose media, the *L. lactis noxE* gene and *C. tropicalis PDC1* gene were introduced to BD5X-TAL1-mXR. The resulting strain, BD5X-TAL1-mXR-nox-pdc produced 11.3 g/L of 2,3-BD from xylose with 0.35 $g_{2,3\text{-BD}}$ /

g_{xylose} of yield and 0.47 g/L/h of productivity in the batch fermentation. In the fed-batch fermentation without addition of ethanol, the BD5X-TAL1-mXR-nox-pdc strain produced 69.2 g/L of 2,3-BD from xylose with 0.38 $g_{2,3\text{-}BD}$ / g_{xylose} of yield and 0.28 g/L/h of productivity

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국 문 초 록

2,3-Butanediol (2,3-BD)는 활용도가 높은 화학 소재로 화학적 생산이 가능하지만 바이오 기술이 개발됨에 따라 생물공학적으로 생산이 가능해졌다. 박테리아를 이용하여 고수율로 생산할 수 있지만 대부분 병원성 박테리아로 분류된다. 따라서 화장품 원료와 생합성 고무의 전구체로 이용되는 2,3-BD 안전과 산업화 측면에서 대량생산공정 구축이 어렵다. 그 대안으로 GRAS (Generally Recognized As Safety) 미생물로서 알려진 *Saccharomyces cerevisiae*가 이용될 수 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.

환경문제를 비롯한 온실가스 감축과 지속 가능한 발전이 대두되고 있는 시점에서, 석유를 기반으로 하는 화학 산업에서 바이오매스를 이용하는 화학 산업이라는 새로운 패러다임이 제시되고 있다. 이러한 바이오 화학 산업의 적용을

위하여 섬유소계 바이오매스를 활용한 미생물학적 생산방법이 주목받고 있다. 그러나 섬유소계 바이오매스에 다량 포함되는 오탄당인 자일로스에 대한 2,3-BD 생산 연구는 포도당ㅇ,ㄹ 활용한 연구와 비교하여 부족하다.

따라서 본 연구에서는 효모를 이용하여 자일로스로부터 2,3-BD 생산성을 향상하고 글리세롤 생성을 감소시키는 것을 목표로 실험을 수행했다. 선행연구자에 의해 pyruvate decarboxylase 활성이 완전히 제거되고 2,3-BD 대사 경로가 도입된 효모 (BD5)와 pyruvate decarboxylase 활성 제거 후 진화공법을 통해 포도당 신호 조절자의 역할을 하는 *MTH1* 유전자의 아미노산 염기서열에 돌연변이가 발생한 효모에 자일로스 대사관련 유전자를 도입한 효모 (BD4X)가 제작된 바 있다. 본 연구에서는 돌연변이가 일어나지 않은 균주 (BD5)에 자일로스 대사관련 유전자를 도입한 효모 (BD5X)를 구축하고, 회분식 배양하여 선행연구자가 구축한 균주 (BD4X)와 비교한 결과 생산성이 16% 증가함을 확인하였다.

두 번째로, 자일로스 소모 속도를 증진시키기 위해 오탄당인산화 경로 내에서 속도조절단계에 관여하는 효소로 알려진 transaldolase를 암호화하는 *Scheffersomyces stipitis* 유래의 *TAL1*과, *S. stipitis* 유래의 자일로스를 자일리톨로 전환시키는 xylose reductase를 암호화 하며 보효소로 NADH를 선호하는 돌연변이 *XYL1*을 도입한 효모 (BD5X-TAL1-mXR)를 제작하였다. 회분식 배양 결과, 이전 효모 (BD5X)와 비교하여 2,3-BD 생산성이 약 66% 증가함을 확인할 수 있었다.

세 번째로, pyruvate decarboxylase 결여 효모의 단점인 탄소원에 의존적인 생장 체계를 극복하고, 보효소 불균형으로 인해 부산물로 글리세롤이 다량 축적되는 문제점을 보완하고자 선행연구자에 의해 확인된 *Lactococcus lactis* 유래의 *noxE* 유전자를 도입한 균주 (BD5X-TAL1-mXR-nox)와 이 균주에 *Candida tropicalis* 유래의 *PDC1* 유전자를 도입한 균주 (BD5X-TAL1-mXR-nox-pdc)를 구축하였다. 균주 (BD5X-TAL1-mXR-nox-pdc) 회분식 배양 결과, 비교군 (BD5X-TAL1-mXR)과 비교하여약 48% 감소한 글리세롤 수율을 확

인할 수 있었으며, 추가적으로 에탄올을 공급하지 않고 회분식 배양한 결과 탄소원에 비의존적인 생장이 가능함을 확인하였다. 이 균주의 발효 능력을 정확히 확인하기 위하여 유가식 배양을 수행하였고, 최종적으로 자일로스로부터 69.2 g/L의 2,3-BD가 생성되며 2,3-BD 수율은 0.38 $g_{2,3\text{-}BD}/g_{xylose}$이었다. 주요 부산물인 글리세롤은 13.8 g/L 생성되었으며, 수율은 0.08 $g_{glycerol}/g_{xylose}$로 매우 낮은 수치를 보였다. 이와 같은 2,3-BD 농도는 선행연구에서 구축한 BD4X 균주를 이용하여 포도당과 자일로스 혼합당원으로부터 유가식발효를 통해 생산된 2,3-BD 농도인 43.6 g/L보다 58% 증가한 것이다.

이를 통하여 재조합 *S. cerevisiae*인 BD5X-TAL1-mXR-nox-pdc 균주는 섬유소계 바이오매스에 다량 포함되는 오탄당인 자일로스로부터 2,3-BD를 효율적으로 생산할 수 있는 균주임을 증명하였다.

주요어 : 2,3-Butanediol (2,3-BD), pyruvate decarboxylase 결여 효모, 자일로스, 목질계 바이오매스, 유가식배양, 보효소 불균형, 알돌 전이 효소

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