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농학박사학위논문

대사공학적으로 설계된 재조합 효모를 이용한 목질계 바이오매스로부터 2,3-butanediol 생산

Production of 2,3-butanediol from cellulosic biomass by metabolically engineered S*accharomyces cerevisiae*

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Advisor: Professor Jin-Ho Seo

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ABSTRACT

2,3-Butanediol (2,3-BD) is a platform chemical with wide industrial applications. Most microbial fermentations for 2,3-BD production have been focused on pathogenic bacteria, which makes large-scale fermentation difficult in terms of safety and industrialization.

Since *Saccharomyces cerevisiae*, a popular GRAS (Generally Recognized As Safe) microorganism, is known to produce a trace amount of 2,3-BD naturally, the baker's yeast was metabolically engineered for efficient production of 2,3-BD by introducing the 2,3-BD metabolic pathways and by modulating the central carbon metabolism. A fed-batch fermentation strategy was optimized in order to enhance a final 2,3-BD concentration. To intensify the 2,3-BD biosynthetic pathway, the *alsS* gene encoding α-acetolactate synthase and the *alsD* gene encoding α-acetolactate decarboxylase both from *Bacillus subtilis* and the endogenous *BDH1* gene coding for 2,3-BD dehydrogenase were overexpressed in the wild-type *S. cerevisiae* (D452-2). The resulting strain of *S. cerevisiae* BD0 showed approximately a 10-fold increase in 2,3-BD production compared to the wild strain, but still produced unfavorable ethanol as a major metabolite.

To increase 2,3-BD production through eliminating ethanol production, a pyruvate decarboxylase (Pdc)-deficient mutant (SOS4) was used as a

host for 2,3-BD production. The SOS4 strain grew in a glucose medium and accumulated pyruvate from glucose, a key intermediate for 2,3-BD, without ethanol production. When the *alsS* and *alsD* genes from *B. subtilis* and the endogenous *BDH1* gene were overexpressed in the SOS4, the resulting strain (BD4) not only produced 2,3-BD with a high yield of 0.34 g 2,3-BD/g glucose, but also consumed glucose faster than the parental strain. In a fed-batch fermentation under the optimum aeration condition, 2,3-BD concentration increased up to 96.2 g/L from glucose.

The use of xylose that is abundant in lignocellulosic hydrolyzate would make the production of 2,3-BD more sustainable and economical. However, *S. cerevisiae* cannot ferment xylose as a sole carbon source. For xylose utilization, the *XYL1*, *XYL2*, and *XYL3* genes coding for xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) derived from *Scheffersomyces stipitis* were introduced into the SOS4 strain. The resulting strain (SOS4X) accumulated pyruvate by using xylose without ethanol production. Additionally, the *alsS* and *alsD* genes from *B. subtilis* and the endogenous *BDH1* gene were overexpressed in the SOS4X for production of 2,3-BD from xylose. As a result, the resulting strain (BD4X) produced 20.7 g/L 2,3-BD with a yield of 0.27 g 2,3-BD/g xylose, showing that (*R*, *R*)-2,3-BD was dominantly produced. The titer of 2,3-BD from xylose increased up to

43.6 g/L in a fed-batch fermentation. These results suggest that *S. cerevisiae* might be a promising host for producing 2,3-BD from lignocellulosic biomass for industrial applications.

Key words: 2,3-Butanediol (2,3-BD), pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae*, xylose, lignocellulosic biomass, fed-batch fermentation

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Characteristics and microbial production of 2,3-butandiol

1.1. 2,3-Butanediol

2,3-Butanediol (2,3-BD) is known as 2,3-butylene glycol, 2,3-dihydroxybutane, or dimethylethlene glycol with a molecular weight of 90.12 kDa (Syu, 2001). 2,3-BD can serve as an “octane booster” for gasoline owing to a high octane number and an antifreeze agent (especially, (*R*,*R*)-form of 2,3-BD) due to its low freezing point of -60°C (Celinska and Grajek, 2009). Especially, 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. Acetoin and diacetyl produced by dehydrogenation of 2,3-BD can be used as flavoring agents, cosmetic and food products (Bartowsky and Henschke, 2004; Soltys et al., 2001). The ketaliztion 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 drugs, cosmetics, and plasticizers (Garg and Jain, 1995). Dehydration of 2,3-BD yields methyl ethyl ketone (MEK) and 1,3-butadiene. MEK is an effective fuel additive and can also be used as a solvent for resins and lacquers (Flickinger, 1980; van Haveren et al., 2008).

The most promising application of 2,3-BD is its conversion to 1,3-butanediene which is a monomer for the production of synthetic rubber

(van Haveren et al., 2008). The world market for 1,3-butadiene is largely driven by demand for elastomers due to their extensive use in tire manufacturing. The global market for 1,3-butadiene was estimated at USD 22 billion for 2011, and is expected to rise by 45.6% in the next 5 years and reach a market size of approximately 32 billion by 2018 (http://www.transparencymarket research.com). According to the increase of 1,3-butadiene market, the manufacturing of 2,3-BD is still growing by an annual rate of 4–7% (Syu, 2001).

Biological production of 2,3-BD has been studied for more than 100 years. Microbial production of 2,3-BD is summarized in Table 1. The microbial production started in fermentations using *Klebsiella pneumonia* and *Paenibacillus polymyxa* (Donker, 1926; Harden and Walpole, 1906). As previously mentioned, conversion of 2,3-BD into 1,3-butandien is an encouraging route. Therefore demands for 1,3-butadiene caused by the increasing need for synthetic rubber during World War II stimulated intense interests in microbial 2,3-BD production, resulted in the development of pilot-scale operations. However, less expensive routes for chemical production of 1,3-butadiene from petroleum caused a decline of microbial 2,3-BD production (Celinska and Grajek, 2009). In the 1970s, the long-term prospects of rising petroleum prices revived significant interests in producing 2,3-BD using microbial fermentations from biomass (Voloch

et al., 1985). Recently, microbial 2,3-BD production from renewable biomass has attracted great attention in economic and environmental aspects (Li et al., 2010b).

2,3-BD exists in three stereoisomeric forms: levo-[D-(−)-, (*R*,*R*)-], dextro-[L-(+)-, (*S*,*S*)-], and meso-forms (Fig. 1). The levo- and dextro-forms are optically active, while the meso- form shows optically inactive (Syu, 2001). As it is complicated and expensive to selectively produce stereoisomers through chemical processes, a biological route through microbial fermentations is preferable to obtain pure stereoisomers of 2,3-BD (Liu et al., 2011; Ui et al., 2004). As described in Table 1, steroisomers are dependent on the microorganisms used, and a mixture of two stereoisomers is generally formed (Maddox, 1996). For example, *Klebshiella* sp. and *Enterobacter aerogens* produce meso-2,3-BD and (*S*,*S*)-2,3-BD. *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* also produce a mixture of (*R*,*R*)-2,3-BD and meso-2,3-BD (Celinska and Grajek, 2009). On the other hand, *P. polymyxa* and *Serratia marcescens* have the ability to produce (*R*,*R*)-2,3-BD and meso-2,3-BD in the pure form, respectively. (Ji et al., 2011; Zhang et al., 2010b). Until now, the mechanisms underlying the formation of stereoisomers were not fully understood (Ji et al., 2011).

1.2. Biosynthesis of 2,3-BD in bacteria

Many bacteria can produce 2,3-BD, but only a few might be regarded as 2,3-BD producers (Garg and Jain, 1995). Up to date, *K. pnumoniae*, *K. oxytoca*, and *P. polymyxa* are unbeatable as 2,3-BD producers with high yield and productivity. *E. aerogenes* and *S. marcescens* are also considered to be promising microorganisms (Celinska and Grajek, 2009; Ji et al., 2011).

In bacteria, 2,3-BD is produced via a mixed acid fermentation along with byproducts such as ethanol, acetate, lactate, formate and succinate (Maddox, 1996; Magee and Kosaric, 1987). Three key enzymes are involved in 2,3-BD production from pyruvate. As shown in Fig. 2, pyruvate formed from glycolysis can be converted into α-acetolactate catalyzed by α-acetolactate synthase (ALS). Sequentially, α-acetolactate can be converted to acetoin by α-acetolactate decarboxylase (ALD) under anaerobic conditions. If oxygen is present, α-acetolactate can undergo spontaneous decarboxylation producing diacetyl. Then, diacetyl reductase (DAR), also known as acetoin dehydrogenase, can convert diacetyl to acetoin. Finally, 2,3-BD is produced from the reduction of aceoin by butanediol dehydrogenase (BDH) which is also known as acetoin reductase (AR) (Celinska and Grajek, 2009).

Most bacteria used 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. Although *P. polymyxa* is of great interest as being a nonpathogenic microorganism, a lack of genetic tools available for genetic manipulation of *P. polymyxa* has prevented the microorganism from metabolic engineering for the development of 2,3-BD production (Kim and Timmusk, 2013; Li et al., 2013).

1.3. Biosynthesis of 2,3-BD in *Saccharomyces cerevisiae*

S. cerevisiae is known to produce a trace amount of 2,3-BD naturally, but the yield and productivity of 2,3-BD are very low. Therefore wild-type *S. cerevisiae* cannot be considered as a 2,3-BD producer (Garg and Jain, 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 (Fig. 3). While pyruvate can be converted into α-acetolactate by α-acetolactate synthase (Ilv2) involved in the isoleucine and valine synthetic pathway, α-acetolactate cannot be enzymatically decarboxylated into acetoin as acetolactate decarboxylase is not present in *S. cerevisiae* unlike 2,3-BD producing

bacteria. α-Acetolactate can be converted into diacetyl by spontaneous decarboxylation in the presence of oxygen in the similar manner of the bacteria. Diacetyl produced is sequentially reduced into acetoin by diacetyl reductase. Alternatively, pyruvate decarboxylase is known to condense pyruvate or acetaldehyde into acetoin. Finally, acetoin is converted into 2,3-BD by butanediol dehydrogenase (Bdh1) (Gonzalez et al., 2010). In general, 2,3-BD accumulated at concentrations ranging from 0.2 to 3.0 g/L during wine fermentation (Ehsani et al., 2009; Guymon and Crowell, 1967).

2,3-BD production by *S. cerevisiae* has not been done extensively. Most previous attempts focused on not producing 2,3-BD as the main product but improving a flavor in beer and wine fermentations. Diacetyl and acetoin produced in a brewing process is undesirable due to their unpleasant flavor and low taste threshold (0.02–0.10 mg/L and 1.00–1.50 mg/L, respectively). On the other hand, 2,3-BD has a higher flavor threshold than diacetyl and acetoin. Some studies successfully reduced the diacetyl production in a brewer's yeast by expressing heterologous α-acetolactate decarboxylase from *K. terrigena* and *E. aerogenes*, resulting in an increase in 2,3-BD production (Blomqvist et al., 1991; Suihko et al., 1990). Overexpressing the *BDH1* gene coding for native NADH-dependent Bdh1 or NADPH-dependent *BDH1* mutant gene contributed to an enhancement of 2,3-BD production by

facilitating the conversion of acetoin into 2,3-BD. Recently, research focused on 2,3-BD production by engineered *S. cerevisiae* was reported. A strategy for gene deletion was performed by using *in silico* genome-scale metabolic analysis. Deletion of *ADH1*, *ADH3*, and *ADH5* genes coding for alcohol dehydrogenase resulted in an improvement of 2,3-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 in a fermentation by the engineered *S. cerevisiae* deficient in the *ADH* genes in anaerobic conditions (Ng et al., 2012).

1.4. Pyruvate decarboxylase-deficient *S. cerevisiae*

S. cerevisiae, which has been traditionally used to ferment sugar to ethanol, is a good choice as a host for the production of therapeutic proteins or chemicals by metabolic engineering. Because the yield of the desired product should be maximized in respect of the economy, it is necessary to redirect carbon fluxes away from ethanol production towards the desired product. In order to minimize ethanol production and to maximize the production of desired compounds, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* has been employed 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. In *S. cerevisiae*, there are three structural genes (*PDC1*, *PDC5*, and *PDC6*) encoding active pyruvate decarboxylase isoenzymes. Among these genes, disruption of *PDC1* and *PDC5* or all three *PDC* genes led to elimination of pyruvate decarboxylase activity completely (Flikweert et al., 1996).

The Pdc-deficient strains have potential defects for industrial fermentations. Firstly, the Pdc-deficient mutant needs external supplement of two-carbon compounds (C_2) 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 via acetate in the cells. However, synthesis of acetaldehyde is blocked due to the elimination of Pdc activity, which leads to the shortage of cytosolic acetyl-CoA. Secondly, the Pdc-deficient mutant 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. Recently, a C_2-independent and glucose-tolerant Pdc-deficient strain (TAM) was constructed by laboratory evolution (van Maris et al., 2004). Internal deletion in the *MTH1* gene coding for a negative regulator of the glucose-sensing pathway was identified in the TAM strain by which a phosphorylation site and putative PEST regions were missed (Oud et al., 2012). Also, a C_2-independent and glucose-tolerant Pdc-deficient strain (SOS4) was also constructed. 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 (Ala81Pro), unlike the TAM strain which had internal deletion in the *MTH1* gene (Fig. 4) (Kim et al., 2013a).

Without extracellular glucose, Mth1 represses the transcription of the *HXT* genes together with a transcriptional regulator of Rgt1. When glucose is present, Mth1 is phosphorylated by the casein kinase I (Yck1/2) activated by the signal from the Rgt2/Snf3 glucose sensors, thereby Mth1 is degraded. 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 (Fig. 5) (Kim, 2009; Lafuente et al., 2000; Moriya and Johnston, 2004). However, the Mth1 mutation did not execute the de-repression of hexose transporters. Therefore, glucose uptake rate of the Mth1 mutants

cannot be elevated according to external glucose concentrations. The internal deletion or point mutation of the *MTH1* gene in the TAM and SOS4 strains was likely to reduce glucose uptake, which alleviated the pyruvate accumulation and redox imbalance from eliminating Pdc activity in the evolved Pdc-deficient strains (Kim et al., 2013a).

1.5. Objectives of the dissertation

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

1) Production of 2,3-BD from glucose by introducing heterologous *alsS* and *alsD* genes and overexpressing endogenous *BDH1* gene involved in the 2,3-BD biosynthetic pathway,
2) Improvement of 2,3-BD production by modulation of the ethanol biosynthetic pathway in *S. cerevisiae*,
3) Development of engineered *S. cerevisiae* able to produce 2,3-BD from a mixture of glucose and xylose,
4) Optimization of fermentation process for efficient production of 2,3-BD for cellulosic biomass by engineered *S. cerevisiae*.

Table 1. Microbial production of 2,3-butanediol (2,3-BD)

Strain	Stereo-isomer	Substrate	Method	2,3-BD			Characteristics	Reference
				Titer (g/L)	Yield (g/g)	Productivity (g/L-h)		
Klebsiella pneumoniae	meso-, L-(+)-	Glucose	Fed-batch	150.0	0.40	3.95	*K. pneumonia* SDM by ion beam mutation	(Ma et al., 2009)
		Glucose	Fed-batch	92.4	0.43	1.84	Supplementary of $(NH_4)_2BPO_4$	(Qin et al., 2006)
		Glycerol	Fed-batch	70.0	0.39	0.47	Optimization of conditions (pH)	(Petrov and Petrova, 2010)
		Corncob molasses	Fed-batch	78.9	0.39	1.29	*Klebsiella pneumonia* SDM	(Wang et al., 2010)
		Jerusalem artichoke	Fed-batch	84.0	0.29	2.10	SSF	(Sun et al., 2009)
Klebsiella oxytoca	meso-, L-(+)-	Glucose	Fed-batch	130.0	0.47	1.62	Deletion of *aldA*	(Ji et al., 2010)
		Glucose	Fed-batch	85.5	0.47	3.00	Effects of aeration on 2,3-BD production	(Qureshi and Cheryan, 1989)
		Molasses	Fed-batch	118.0	0.41	2.35	Cell recycling fermentation	(Afschar et al., 1991)
		Corncob hydrolysate	Fed-batch	35.7	0.50	0.59	Effect of acetate supply on 2,3-BD production	(Cheng et al., 2010)
		Corncob cellulose	Batch	25.0	0.31	0.36	Supplementation of cellulose (SSF)	(Cao et al., 1997)

(be continued)

Strains	Stereo-isomer	Substrate	Methods	2,3-BD			Characteristics	Ref.
				Titer (g/L)	Yield (g/g)	Productivity (g/L-h)		
Enterobacter aerogens	meso-, L-(+)-	Glucose	Fed-batch	110.0	0.49	5.40	Cell recycling fermentation	(Zeng et al., 1991)
Serratia marcescens	Meso-	Sucrose	Fed-batch	152.0	0.41	2.67	Serrawettin-deficient mutant	(Zhang et al., 2010a)
Paenibacillus polymyxa	D-(-)-	Sucrose	Fed-batch	111.0	-	2.05	Fed-batch fermentation	(Hassler et al., 2012)
		Jerusalem artichoke	Batch	36.9	0.50	0.88	One-step fermentation of raw inulin	(Gao et al., 2010)
Bacillus subtilis	D-(-)-Meso-	Glucose	Batch	2.5	0.31	0.27	Effect of oxygen on 2,3-BD production	(Moes et al., 1985)
Bacillus licheniformis	D-(-)-Meso-	Glucose	Batch	8.7	0.12	0.47	Batch cultivation	(Nilegaonkar et al., 1992)
Bacillus amyloliquefaciens	D-(-)-Meso-	Glucose	Fed-batch	92.3	0.42	0.96	GRAS strain	(Yang et al., 2011)
Escherichia coli	Meso-	Glucose	Batch	13.0	0.43	0.2	Δ*idhA*, *pta*, *adhE*, *poxB* and Overexpression of *alsS_alsD* (*B. subtilis*), and *budC* (*K. pneumonia*)	(Li et al., 2010c)
Saccharomyces cerevisiae	D-(-)-Meso-	Glucose	Batch	7.4	0.03	0.06	Δ*ald6* and overexpression of *GPD1*, $BDH1^{m}$ genes	(Ehsani et al., 2009)
	D-(-)-Meso-	Glucose	Batch	2.3	0.11	0.03	Δ*adh1,3,5* genes	(Ng et al., 2012)

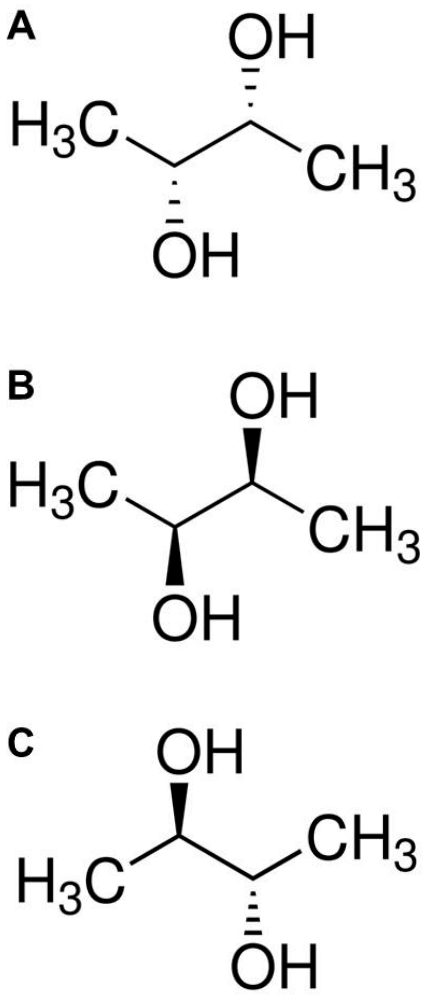


Fig. 1. The stereoisomers of 2,3-butanediol (2,3-BD).

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

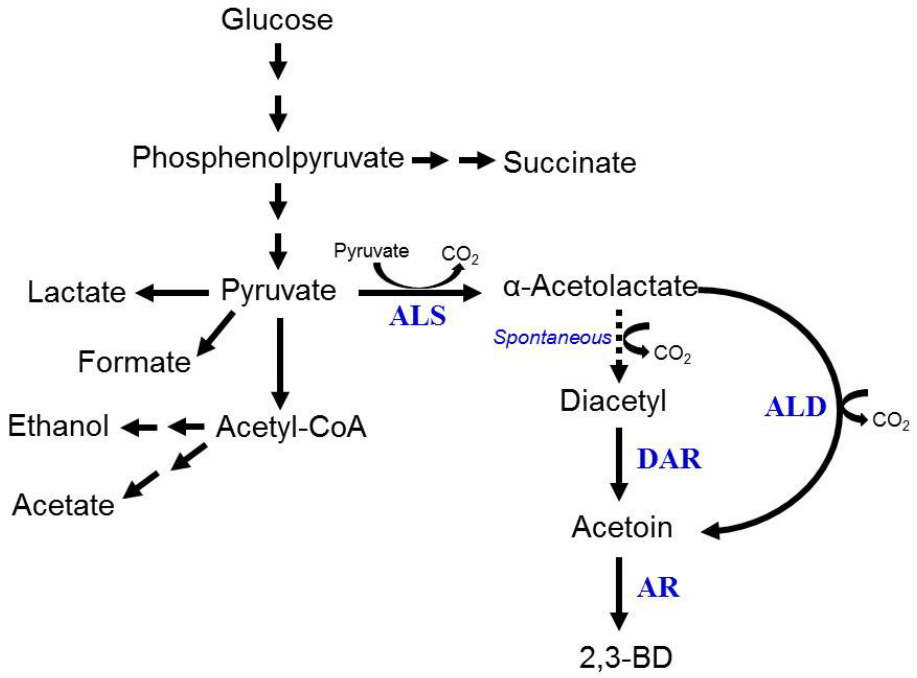


Fig. 2. Mixed acid-2,3-BD pathway in bacteria.

The names of enzymes are abbreviated as follows: ALS (α-acetolactate synthase), ALD (α-acetolactate decarboxylase), DAR (Diacetyl reductase), and AR (Acetoin reductase).

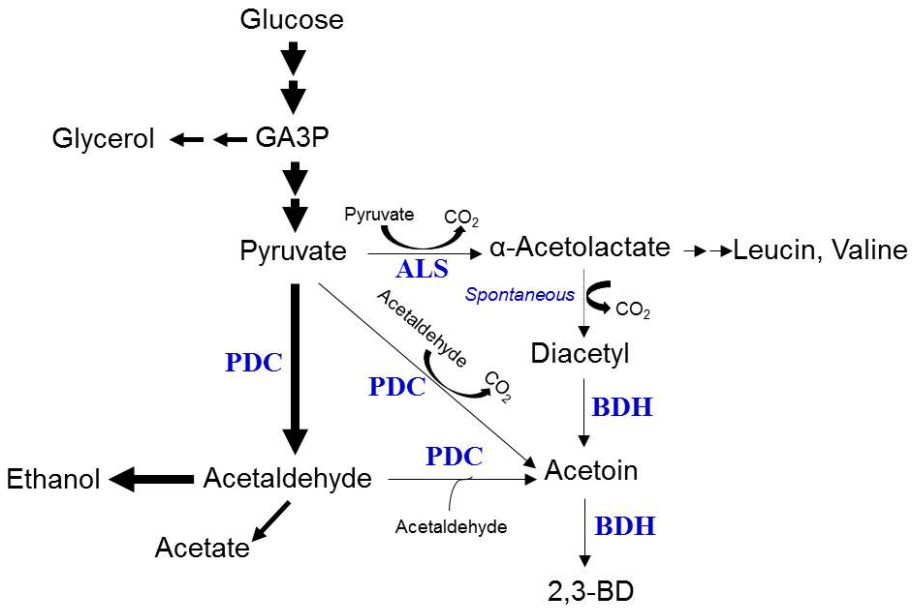


Fig. 3. 2,3-BD biosynthetic pathway in *Saccharomyces cerevisiae.*

The name of enzymes are abbreviated as follows: ALS (α-acetolactate synthase), PDC (Pyruvate decarboxylase), and BDH (2,3-BD dehydrogenase).

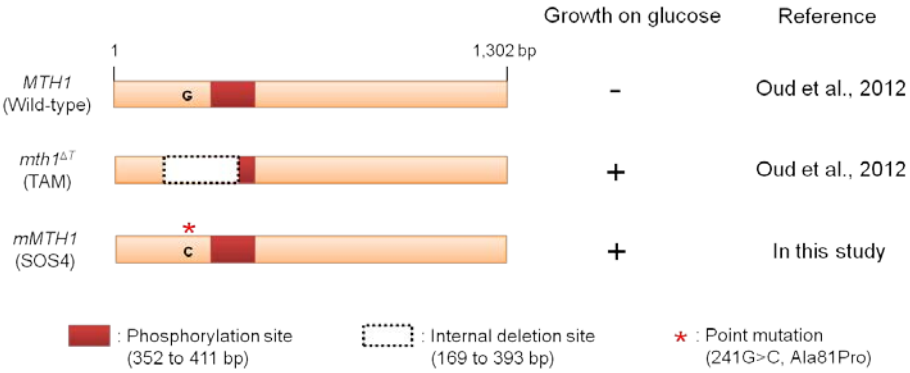


Fig. 4. Comparison of the isolated mutant allele of *MTH1* gene from two Pdc-deficient mutants (TAM and SOS4) exhibiting glucose tolerance (Kim et al., 2013a).

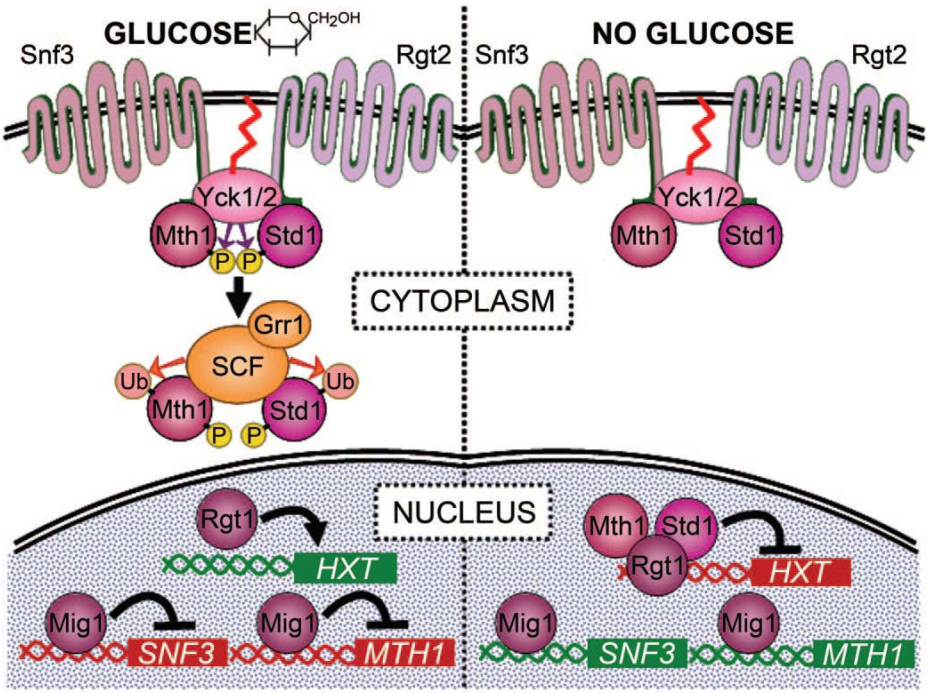


Fig. 5. The Snf3/Rgt2 signaling pathway (Santangelo, 2006).

Chapter 2

Production of 2,3-butanediol (2,3-BD) through complementing the 2,3-BD biosynthetic pathway of *Saccharomyces cerevisiae*

2.1. Summary

2,3-Butanediol (2,3-BD) is a platform chemical with wide industrial applications. *Saccharomyces cerevisiae* is able to synthesize 2,3-BD naturally, but the amount of 2,3-BD is much lower than that of 2,3-BD producing bacteria. In order to produce 2,3-BD efficiently in *S. cerevisiae*, the 2,3-BD biosynthetic pathway was complemented. The heterologous *alsS* gene encoding α-acetolactate synthase and *alsD* gene coding for α-acetolactate decarboxylase from *Bacillus subtilis* were introduced into *S. cerevisiae*. In addition, the innate *BDH1* gene coding for 2,3-BD dehydrogenase was overexpressed in *S. cerevisiae*. The resulting strain (BD0) showed a 10-fold improvement in 2,3-BD production compared to the wild strain. Also, the *PDC1*-deficient mutant was chosen to enforce the inefficient acetoin flux to 2,3-BD metabolism. The resulting strain (BD1) exhibited a two-fold improvement in 2,3-BD production in comparison with the BD0 strain. However, ethanol was inevitably produced as a major metabolite, suggesting that elimination of ethanol production is necessary for efficient production of 2,3-BD.

2.2. Introduction

2,3-Butanediol (2,3-BD) is a platform chemical with wide industrial applications that can be used to produce other chemicals and synthesize diverse products, such as drugs, cosmetics, and industrial solvents (Celinska and Grajek, 2009; Garg and Jain, 1995; Syu, 2001). It is known that many bacterial species synthesize 2,3-BD. Among these bacteria, *K. pneumoniae*, *K. oxytoca* and *P. polymyxa*, and *E. aerogenes* are considered to be promising microorganisms for industrial applications (Perego et al., 2000). While these bacteria are capable of producing 2,3-BD with high yields and productivities, most of them are classified as pathogen bacteria so that large-scale fermentations might be difficult in terms of safety and industrialization. As alternative, 2,3-BD production by a GRAS (Generally Recognized As Safe) microorganism would be desirable. In this context, *S. cerevisiae* is appropriate even though the baker's yeast produce 2,3-BD with low yield and productivity (Garg and Jain, 1995).

Although two innate 2,3-BD pathways exit in *S. cerevisiae*, the metabolic fluxes toward acetoin are not high enough to achieve efficient production of 2,3-BD. Firstly, *S. cerevisiae* α-acetolactate synthase (Ilv2) has a lower affinity for pyruvate than bacterial α-acetolactate synthases. Moreover, *S. cerevisiae* does not possess α-

acetolactate decarboxylase (ALDC) unlike the 2,3-BD-producing bacteria, so α-acetolactate cannot be enzymatically decarboxylated into acetoin in *S. cerevisiae* (Dulieu and Poncelet, 1999; Yamano et al., 1994). Secondly, pyruvate decarboxylase (Pdc) carries out carboligase reactions, resulting in the formation of acetoin from acetaldehyde, but the reaction counts for only 1% of the total Pdc catalyzing reaction (Sergienko and Jordan, 2001). Therefore, it is necessary to amplify the metabolic fluxes from pyruvate to 2,3-BD via α-acetolactate.

In this Chapter, tolerance of *S. cerevisiae* on 2,3-BD was evaluated and the efficient 2,3-BD biosynthetic pathway was introduced into *S. cerevisiae* by overexpression of heterologous and endogenous genes essential for 2,3-BD production.

2.3. Materials and Methods

2.3.1. Strains and plasmids

Strains and plasmids used in this Chapter are summarized in Table 2. *S. cerevisiae* D452-2 was used as a host for 2,3-BD production. For 2,3-BD production, three plasmids of pRS426_alsS, pRS423_alsD, and pRS425_BDH1 contained the *alsS*, *alsD*, and *BDH1* genes, respectively, of which expression was under the control of a constitutive GPD promoter. *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and manipulation.

2.3.2. Medium and culture conditions

E. coli was grown in Lysogeny Broth (LB) medium with 50 μg/mL ampicillin when required. Yeast strains were cultivated at 30°C in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) with 20 g/L glucose. To select pre-culture transformants using an amino acid auxotrophic marker, yeast synthetic complete (YSC) medium was used. The YSC medium contained 6.7 g/L yeast nitrogen base (YNB), 20 g/L glucose, and appropriate nucleotides and amino acids.

2.3.3. Yeast transformation

Transformation for introducing and overexpressing genes involved in

the 2,3-BD biosynthetic pathway in *S. cerevisiae* was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). Transformants were selected on YSC medium containing 20 g/L glucose. Amino acids and nucleotides were added as necessary.

2.3.4. Fermentation experiments

Seed cultures were prepared by culturing in 5 mL of YSC medium containing 20 g/L glucose. Yeast cells were harvested at a mid-exponential phase and inoculated into main cultures with initial OD_{600} of ~1.0. Batch fermentations were performed in 50 mL of YP medium with 20 g/L glucose in a 250 mL flask at 30°C. 80 rpm of agitation speed was set for oxygen-limited condition.

2.3.5. Analytic methods

Cell growth was monitored by optical density (OD) at 600 nm using a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and dry cell weight (DCW) was estimated by using a conversion factor: DCW (g/L) = OD * 0.3 (Oh et al., 2012). Glucose, pyruvate, glycerol, acetate, acetoin, 2,3-butanediol (2,3-BD), and ethanol concentrations were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) with a Bio-Rad Aminex HPX-87H column. The column was eluted with 5

mM sulfuric acid at a flow rate of 0.6 ml/min at 60°C. Pyruvate was detected by a UV detector at 214 nm and the others were detected by a refractive index detector. All reagents including (*R*, *R*)-2,3-BD for standard solution were purchased from Sigma-Aldrich.

2.4. Results

2.4.1. 2,3-BD tolerance test of *S. cerevisiae*

To investigate whether *S. cerevisiae* is appropriate as a 2,3-BD producer, a tolerance test to 2,3-BD was performed under various concentrations of 2,3-BD. *S. cerevisiea* D452-2 was aerobically cultured in YP medium with 20 g/L glucose (YPD20) containing 2–12 % of 2,3-BD. The tolerance value was evaluated by measuring specific growth rate, dry cell weight (DCW), and glucose consumption rate at the point of glucose depletion. The results showed that the detrimental effect of 2,3-BD on cell growth and glucose consumption rate was moderate up to a concentration of 4 % (Fig. 6). At the concentrations above 4 %, the specific growth rate, DCW and the glucose consumption rate were gradually declined. When cultured in YPD20 medium with 12 % of 2,3-BD, the specific growth rate and glucose consumption rate were reduced by approximately 50 % and 40 %, respectively, in comparison with the case without 2,3-BD.

2.4.2. Construction of the efficient 2,3-BD biosynthetic pathway of *S. cerevisiae*

In order to intensify the 2,3-BD biosynthetic pathway, the *alsS* gene encoding α-acetolactate synthase and *alsD* gene coding for α-acetolactate decarboxylase from *Bacillus subtilis* were introduced into *S. cerevisiae* D452-2. Introduction of the two genes was expected to bypass a slow reaction of endogenous 2,3-BD production via diacetyl. Additionally, the endogenous *BDH1* gene coding for 2,3-butanediol dehydrogenase was overexpressed to enforce the acetoin conversion into 2,3-BD. When cultured in YPD20 medium under oxygen-limited conditions, the control strain with mother plasmids (CON0) produced 7.6 g/L ethanol as a major metabolite and a trace amount (< 0.1 g/L) of 2,3-BD (Fig. 7A), whereas the BD0 strain able to overexpress the 2,3-BD biosynthetic genes produced 0.7 g/L 2,3-BD with reduced ethanol production (7.0 g/L) (Fig. 7B)(Table 3).

However, the BD1 strain still produced ethanol as a major product, suggesting that the heterologous 2,3-BD producing pathway could not compete with the endogenous ethanol producing pathway in *S. cerevisiae*.

2.5. Discussion

In order to achieve high yields and high titers, tolerance to high concentrations of the end-product is one of important parameters in choosing a host for the desired products. A decrease in growth rate of *S. cerevisiae* was observed at ethanol concentrations above 6%, suggesting *S. cerevisiae* is a suitable host to produce ethanol. However, *S. cerevisiae* cannot grow at concentrations higher than 2% of butanol and isobutanol. Tolerance to 2,3-BD in *S. cerevisiae* was similar to ethanol and higher than other four-carbon alcohols such as butanol and isobutanol (Gonzalez-Ramos et al., 2013). Therefore high tolerance to 2,3-BD enables *S. cerevisiae* to be a promising host for 2,3-BD production.

Although two innate 2,3-BD biosynthetic pathways exist, the metabolic fluxes from pyruvate toward acetoin are extremely low in *S. cerevisiae*. In this Chapter, in order to accelerate an inefficient and slow reaction, the 2,3-BD biosynthetic pathway was complemented through introduction of the heterologous *alsS* and *alsD* genes from a 2,3-BD producing microbe (*B. subtilis*) and additional overexpression of the innate *BDH1* gene. 2,3-BD production by the resulting strain (BD0) strain increased by a 10-fold in comparison with the control strain (CON0). It was demonstrated that the overexpression of the *alsS*, *alsD*,

and *BDH1* genes might contribute to intensify the 2,3-BD biosynthetic pathway via α-acetolactate, which resulted in an increment of 2,3-BD production.

The other route toward acetoin via acetaldehyde or pyruvate by pyruvate decarboxylase is too weak to achieve sufficient acetoin for 2,3-BD production. Generally, yeast pyruvate decarboxylase can form acetoin and α-acetolactate, but the reaction is minor (1% of the total reaction) compared to production of acetaldehyde which can be converted into ethanol or other metabolites (Sergienko and Jordan, 2001).

Nevertheless, the BD1 strain still produced unfavorable ethanol as a major product. Therefore redirection of the carbon flux away from ethanol toward 2,3-BD is necessary for efficient 2,3-BD production by removing or decreasing pyruvate decarboxylase activities.

Table 2. Strains and plasmids used in Chapter 2

Name	Description	Reference
Strains		
D452-2	*Saccharomyces cerevisiae* (*MATα, leu2, his3, ura3,* and *can1*)	(Hosaka et al., 1992)
Wild CON	D452-2 (pRS426GPD, pRS423GPD, and pRS425GPD)	In this Chapter
BD0	D452-2 (pRS426_AlsS, pRS423_AlsD, and pRS425_BDH1)	In this Chapter
Plasmids		
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)
pRS426_AlsS	pRS426GPD harboring *alsS* gene from *Bacillus subtilis* str.168	(Kim et al., 2013a)
pRS423_AlsD	pRS423GPD harboring *alsD* gene from *B. subtilis* str.168	(Kim et al., 2013a)
pRS425_BDH1	pRS425GPD harboring *BDH1* gene from *S. cerevisiae* D452-2	(Kim et al., 2013a)

Table 3. Summary of batch fermentations by recombinant *S. cerevisiae* strains possessing the 2,3-BD biosynthetic pathway

Strain	DCW (g/L)	2,3-BD concentration (g/L)	Ethanol concentration (g/L)	Glycerol concentration (g/L)	Yield of 2,3-BD (g 2,3-BD/g Glucose)
Wild CON	4.3±0.1	< 0.1	7.6 ± 0.5	0.3 ± 0.1	≒ 0.00
BD0	4.1±0.1	0.7 ± 0.1	6.4 ± 0.1	0.6 ± 0.1	0.04

All products were collected after 12h cultivation

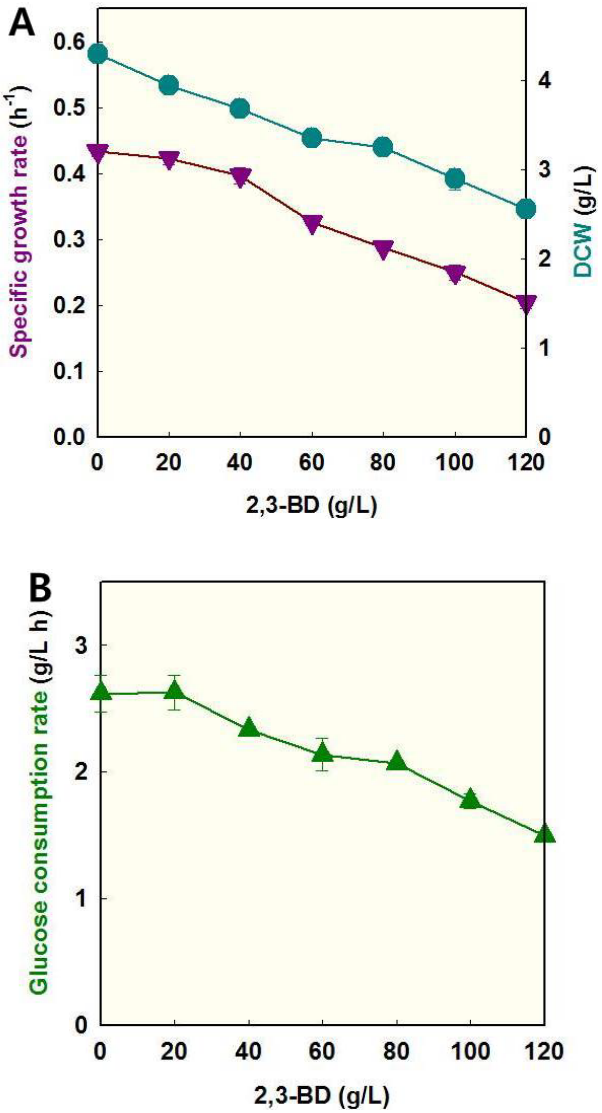


Fig. 6. Specific growth rate and glucose consumption rate of *S. cerevisiae* D452-2 cultured in YPD20 medium containing 2,3-BD concentrations ranging from 0 to 120 g/L. (A) Specific growth rate (▼) and DCW (●); (B) Glucose consumption rate (▲). Error bars represent standard deviations associated with two independent experiments.

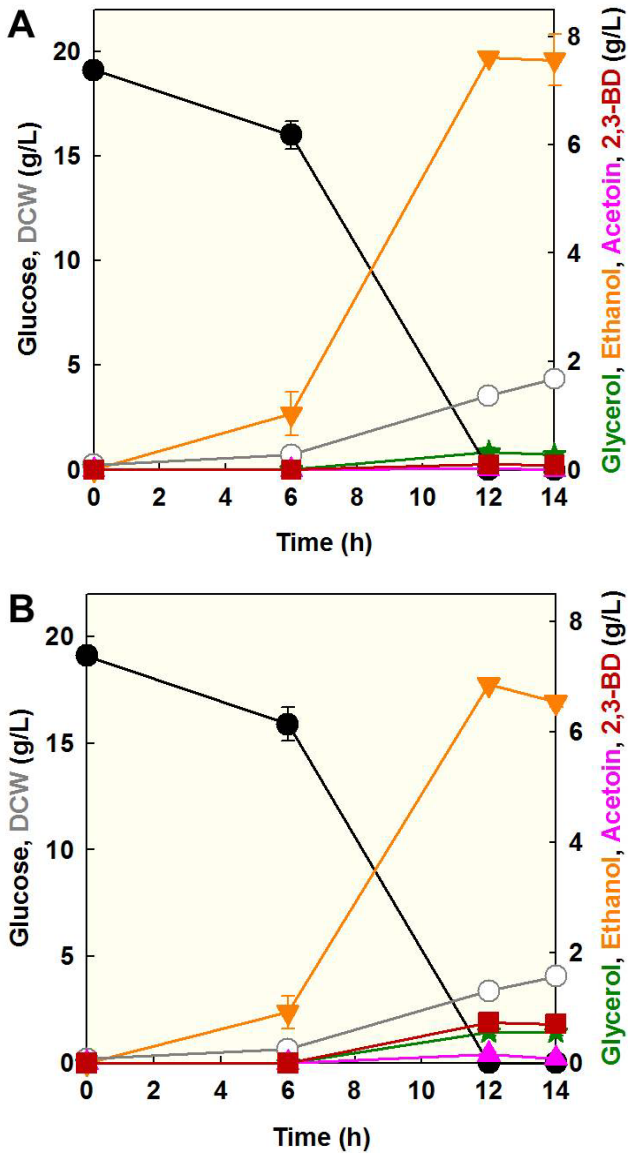


Fig. 7. Batch fermentations of recombinant *S. cerevisiae* D452-2 strain harboring the 2,3-BD biosynthetic pathway. (A) Wild CON (D452-2 with mother vector); (B) BD0 (D452-2 with 2,3-BD pathway). Symbols: Glucose (●), DCW (○), Glycerol (★), Ethanol (▼), Acetoin (▲), and 2,3-BD (■). Error bars represent standard deviations associated with two independent experiments.

Chapter 3

Production of 2,3-butanediol (2,3-BD) by pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*

3.1. Summary

In order to produce 2,3-butanediol (2,3-BD) with a high yield, it is necessary to delete the competing pathway and to overexpress the 2,3-BD biosynthetic enzymes. To this end, the evolved pyruvate-deficient strain (SOS4) was utilized as a host for 2,3-BD production for eliminating ethanol production. Additionally, in order to redirect accumulated pyruvate toward 2,3-BD, the 2,3-BD biosynthetic pathway was intensified through introducing the *Bacillus subtilis alsS* and *alsD* genes and overexpressing the innate *BDH1* gene into the SOS4 strain. The resulting strain (BD4) not only consumed glucose faster than the parental strain (SOS4), but also produced 2,3-BD efficiently. As a result, the BD4 strain was able to produce 34.8 g/L 2,3-BD with a yield of 0.36 g 2,3-BD/g glucose from 100 g/L glucose. To reduce redox imbalance caused by 2,3-BD production, oxygen supply was optimized (300 rpm of agitation speed and 1.0 vvm of aeration). A fed-batch fermentation with optimized conditions resulted in 96.2 g/L 2,3-BD concentration. These results suggested that *S. cerevisiae* might be a promising host for producing 2,3-BD for industrial applications.

3.2. Introduction

Metabolic fluxes towards 2,3-BD in *S. cerevisiae* are not high enough to achieve efficient 2,3-BD production. Ethanol production is the most obvious barrier for efficient 2,3-BD production because pyruvate, a key intermediate, is preferentially used for producing ethanol rather than 2,3-BD in *S. cerevisiae*. An inverse relationship between ethanol and 2,3-BD production in *S. cerevisiae* was obtained through stoichiometric modeling. Disruption of *ADH1*, *ADH3*, and *ADH5* genes coding for alcohol dehydrogenase achieved decent 2,3-BD titer (2.29 g/L 2,3-BD) and yield (0.113 g 2,3-BD/g glucose) by engineered *S. cerevisiae* (Ng et al., 2012).

In order to minimize ethanol production and to maximize 2,3-BD production, a pyruvate decarboxylase (Pdc)-deficient mutant can also be utilized for 2,3-BD production. Pyruvate decarboxylase coded by three structural genes (*PDC1*, *PDC5*, and *PDC6* gene) is located at the branch point between fermentative and respiratory metabolism (Hohmann, 1991). It has been reported that deletion of *PDC1* and *PDC5* genes or all *PDC*s genes cause lack of pyruvate decarboxylase activity (Flikweert et al., 1996).

Two carbon (C_2) compounds such as acetate or ethanol need to be supplemented for Pdc-deficient strains to synthesize lysine and fatty

acid (Flikweert et al., 1999; Pronk et al., 1996). Also, the growth of Pdc-deficient strains on fermentable carbon sources such as glucose is very slow. While respiration is necessary for re-oxidation of cytosolic NADH in the Pdc-deficient strains, glucose represses respiration and makes Pdc-deficient strains suffer from redox imbalance (Pronk et al., 1996). However, C_2-independent and glucose-tolerant Pdc-deficient strains (TAM and SOS4) were constructed by laboratory evolution (Kim et al., 2013a; van Maris et al., 2004). Recently, internal deletion or point mutation of the *MTH1* gene coding for a negative regulator of the glucose-sensing pathway has been identified in the TAM or the SOS4 strain, respectively (Kim et al., 2013a; Oud et al., 2012). The modification of the *MTH1* gene is likely to cause reduced glucose uptake, which mitigated pyruvate accumulation and redox imbalance from eliminating Pdc activity in the evolved Pdc-deficient strain (Kim et al., 2013a; Oud et al., 2012).

Oxygen supply is the most important variable in 2,3-BD production (Celinska and Grajek, 2009). 2,3-BD is produced under low O_2 supply to maintain an internal redox balance during glycolysis and biosynthesis. Generally, NADH formed from glycolysis is regenerated by 2,3-butanediol dehydrogenase in a reversible reaction. Therefore the NAD^+/NADH balance is maintained by relative production of acetoin : 2,3-BD (Voloch M, 1985). Thus, it is essential to establish an optimum

oxygen supply strategy to produce 2,3-BD with high yield and productivity.

Initial attempts to produce 2,3-BD in *S. cerevisiae* through introduction of the bacterial 2,3-BD biosynthetic pathway resulted in trace amounts of 2,3-BD production because of ethanol production as a major metabolite. In this Chapter, the Pdc-deficient *S. cerevisiae* unable to produce ethanol was used as a host for 2,3-BD production (Fig. 8). Also, the effect of oxygen supply on 2,3-BD production in *S. cerevisiae* was investigated. Finally, in order to increase 2,3-BD titer, fed-batch fermentation and cell-recycling fermentation were performed under the optimum aeration condition.

3.3. Materials and Methods

3.3.1. Strains and plasmids

Strains and plasmids used in this Chapter are summarized in Table 4. *S. cerevisiae* D452-2 (*MATα*, *leu2*, *his3*, *ura3*, *can1*) (Hosaka et al., 1992) was used for constructing Pdc-deficient strains (SOS2) which was evolved in laboratory for isolating a Pdc-deficient mutant (SOS4) capable of growing on glucose. For 2,3-BD production, three plasmids (pRS426_alsS, pRS423_alsD, and pRS425_BDH1) containing *alsS*, *alsD*, and *BDH1*, respectively under the control of the constitutive GPD promoter were introduced into the SOS4 strain. *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and manipulation.

3.3.2. Medium and culture conditions

E. coli was grown in Lysogeny Broth (LB) medium with 50 μg/mL ampicillin when required. Yeast strains were cultivated at 30°C in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) with 20 g/L glucose. To select pre-culture transformants using an amino acid auxotrophic marker, yeast synthetic complete (YSC) medium was used. The YSC medium contained 6.7 g/L yeast nitrogen base (YNB), 20 g/L glucose, and appropriate nucleotides and amino acids.

3.3.3. Yeast transformation

Transformation of the 2,3-BD biosynthetic pathway in *S. cerevisiae* was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). Transformants were selected on YSC medium containing 20 g/L glucose. Amino acids and nucleotides were added as necessary.

3.3.4. Fermentation experiments

Seed cultures were prepared by culturing in 5 mL of YSC medium containing 20 g/L glucose. Yeast cells were harvested at a mid-exponential phase and inoculated into main cultures with initial OD_{600} of ~1.0. Batch fermentations were performed in 50 mL of YP medium with 2% or 10% of glucose in a 250 mL flask at 30°C. Either 80 rpm or 250 rpm of agitation speed was set for oxygen-limited or aerobic conditions, respectively.

Fermentations on a bioreactor were performed in 500 mL of YP medium with 100 g/L glucose using a 1 L-bench-top bioreactor (KoBioTech, Korea) at 30°C and pH 5.5. To provide various dissolved oxygen levels, aeration was controlled with combination of air flow rate (0 vvm, 0.25 vvm, and 1.0 vvm) and agitation speeds (300 rpm and 500 rpm).

To obtain a high concentration of 2,3-BD, fed-batch fermentation was

carried out using a bioreactor at 30°C and pH 5.5, yeast cells were harvested at a mid-exponential phase and inoculated into the bioreactor with initial OD_{600} of ~1 or ~10. The initial glucose concentration was 100 g/L glucose. During the repeated batch fermentation, 100 g/L feeding solution of glucose was intermittently supplied when glucose was depleted. 300 rpm of agitation speed and 1.0 vvm of aeration were maintained throughout the cultivation.

For cell recycling fed-batch fermentation, yeast cells were harvested at a mid-exponential phase and inoculated into a bioreactor with initial OD_{600} of ~65. The initial glucose concentration was 100 g/L. During the repeated batch fermentation, 100 g/L feeding solution was intermittently supplied when glucose was depleted. At each point of the glucose supplement, the same volume of culture broth was collected and spun down by centrifugation. The cells were re-inoculated to the bioreactor. 300 rpm of agitation speed and 1.0 vvm of aeration were maintained throughout the cultivation.

3.3.5. Analytic methods

Cell growth was monitored by optical density (OD) at 600 nm using spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and dry cell weight (DCW) was estimated by using a conversion factor: DCW (g/L) = OD * 0.3 (Oh et al., 2012).

Glucose, pyruvate, glycerol, acetate, acetoin, 2,3-butanediol (2,3-BD), and ethanol concentrations were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) with a Bio-Rad Aminex HPX-87H column. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min at 60°C. Pyruvate was detected by a UV detector at 214 nm and the others were detected by a refractive index detector. All reagents including (*R*, *R*)-2,3-BD for standard solution were purchased from Sigma-Aldrich.

3.4. Results

3.4. 1. Confirmation of the evolved Pdc-deficient *S. cerevisiae* (SOS4)

The C_2-independent and glucose-tolerant Pdc-deficient strain (SOS4) was used as a host for 2,3-BD production. Deletion of *PDC 1* and *PDC 5* genes in the SOS4 strain was confirmed by the colony PCR. A shorter PCR size than the wild type was observed in the *PDC* mutant strains (SOS1 and SOS4) (Fig. 9A). Also, plate spotting assay was performed to confirm a C_2-independent and high glucose tolerant phenotype of the SOS4 strain. As described in Fig. 9B, the Pdc-deficient strain (SOS2) was not able to grow in YP medium with either 20 g/L or 100 g/L glucose but the SOS4 strain grew well in YP media with both 20 g/L and 100 g/L glucose.

Cell growth and accumulation of pyruvate by the SOS4 strain on glucose were examined. When cultured in YP medium with 20 g/L glucose as a sole carbon source, the SOS4 strain consumed 20 g/L glucose within 120 h under oxygen-limited conditions (Fig. 10A), without producing ethanol but accumulating 4.5 g/L pyruvate. Under aerobic conditions, the SOS4 strain also accumulated 3.8 g/L pyruvate and showed faster glucose consumption and specific growth rates as

compared to the oxygen-limited conditions (Fig. 10B). The accumulation of pyruvate and elimination of ethanol production by the SOS4 strain during glucose fermentation indicates that Pdc activity in the SOS4 was completely eliminated. However, the SOS4 strain was not able to utilize the glucose supplied. Only 15 or 20 g/L glucose was utilized from the initial 100 g/L glucose under oxygen-limited or aerobic conditions, respectively (Fig. 10C and 10D).

3.4.2. Production of 2,3-BD by the SOS4 strain with the 2,3-BD biosynthetic pathway (BD4)

As the SOS4 strain did not produce ethanol but accumulated pyruvate, the 2,3-BD biosynthetic pathway was introduced into the SOS4 strain for converting accumulated pyruvate into 2,3-BD. The heterologous *alsS* and *alsD* genes encoding α-acetolactate synthase and α-acetolactate decarboxylase were introduced into the SOS4 strain. Additionally, the endogenous *BDH1* gene coding for 2,3-BD dehydrogenase was overexpressed in the SOS4 strain.

As expected, the resulting strain (BD4) produced 2,3-BD without ethanol production, and did not accumulate pyruvate at all under oxygen-limited conditions. Interestingly, the BD4 strain consumed glucose faster than the control strain (CON). While the SOS4 strain with empty plasmids accumulated 4.0 g/L pyruvate in 120 h (Fig. 11A),

the BD4 strain produced 6.4 g/L 2,3-BD in 32 h with rapid glucose consumption (Fig. 11B). These results suggest that accumulated pyruvate in the SOS4 strain can be converted into 2,3-BD by the 2,3-BD biosynthetic pathway with enhanced glucose consumption.

In order to improve final 2,3-BD titer, a batch fermentation with 100 g/L glucose was also carried out under oxygen-limited conditions. The CON strain utilized only 20 g/L glucose supplied to accumulate 4.6 g/L pyruvate (Fig. 11C). However, the BD4 strain was able to consume 100 g/L glucose within 120 h to produce 31.8 g/L 2,3-BD with a high 2,3-BD yield (0.34 g 2,3-BD/g glucose) and volumetric productivity (0.26 g 2,3-BD/L·h) (Fig. 11D). Significant amounts of glycerol also accumulated during the cultivation of the BD4 strain.

2,3-BD production by the BD4 strain was determined under aerobic conditions by increasing agitation speed up to 250 rpm. The BD4 strain produced substantial amounts of acetoin instead of 2,3-BD and accumulated less glycerol when 20 g/L glucose was used under aerobic conditions (Fig. 12A). However, the BD4 strain produced 2,3-BD as a major metabolite when 100 g/L glucose was supplied (Fig. 12B). When 100 g/L glucose was supplied under aerobic conditions, the BD4 strain exhibited faster consumption of glucose, higher cell growth rate, and lower accumulation of glycerol. 2,3-BD was a main fermentation

product and small amounts of acetoin accumulated as well. The results indicated that accumulation of acetoin and glycerol might be controlled by aeration and the concentration of glucose in the medium.

3.4.3. Effect of oxygen supply on 2,3-BD production by the BD4 strain

The maintenance of the internal redox balance is also crucial for minimizing production of other metabolites, such as pyruvate, glycerol, and acetoin (Celinska and Grajek, 2009). Moreover, drastic changes in byproduct formation pattern by the engineered *S. cerevisiae* were detected under different aeration conditions. Therefore, an optimum oxygen supply condition was determined to produce 2,3-BD with reduced byproducts (glycerol and acetoin) formation. In order to determine a quantitative relationship between the oxygen supply and 2,3-BD production, batch cultivations with 100 g/L glucose were performed using a bioreactor under various aeration conditions (0, 0.25, and 1.0 vvm with 300 rpm or 1.0 vvm with 500 rpm) with pH control (pH 5.5). As the supply of oxygen increased from 0.25 vvm at 300 rpm to 1.0 vvm at 500 rpm, faster glucose consumption and cell growth rates were observed (Fig. 13C) (Table 5). Glycerol accumulation reduced, but ratios of acetoin to 2,3-BD increased with higher oxygen supply (Fig. 13A-B). Interestingly, the BD4 strain did not grow well

when aeration was limited, suggesting that the BD4 strain might suffer from redox imbalance under lower aeration conditions (Fig. 14A). Among the tested aeration conditions, 1 vvm of aeration and 300 rpm of agitation speed was optimal for producing 2,3-BD by the BD4 strain using 100 g/L glucose. The BD4 strain produced 34.8 g/L 2,3-BD with the highest yield (0.36 g 2,3-BD/g glucose) and productivity (0.32 g 2,3-BD/L·h) with 1 vvm of aeration and 300 rpm of agitation speed (Fig. 14C).

3.4.4. Fed-batch fermentations and cell-recycling fermentation by the BD4 strain

To determine the potential of the BD4 strain as a 2,3-BD producer, fed-batch fermentations were carried out through intermittent addition of glucose under the optimum aeration condition (Table 6).

The final concentration of 2,3-BD was 96.5 g/L after 362 h cultivation, with a 2,3-BD yield (0.30 g 2,3-BD/g glucose) and volumetric productivity (0.27 g 2,3-BD/L·h) (Fig. 15A). To increase volumetric productivity, initial inoculation OD_{600} was increased up to 10. The final concentration of 2,3-BD was 96.2 g/L after 244 h cultivation, with a 2,3-BD yield (0.28 g 2,3-BD/g glucose) and volumetric productivity (0.39 g 2,3-BD/L·h) (Fig. 15B).

Since the cells act as biocatalyst for 2,3-BD, a cell-recycling fermentation with high cell density was performed. Initial inoculation OD was 60, and cells from culture media was recovered and recycled to a bioreactor along with glucose addition. The final concentration of 2,3-BD was 81.9 g/L after 93 h cultivation, with a 2,3-BD yield (0.27 g 2,3-BD/g glucose) and volumetric productivity (0.88 g 2,3-BD/L·h) (Fig. 16)

3.5. Discussion

Pyruvate, a starting precursor for 2,3-BD biosynthesis, is mainly used for producing ethanol in *S. cerevisiae*, which leads to extremely poor 2,3-BD production. In order to overcome this problem, it is necessary to utilize Pdc-deficient *S. cerevisiae* which accumulates pyruvate because of elimination of Pdc activity. While deletion of the Pdc coding genes (*PDC1* and *PDC5*) causes a C_2 dependence and severe growth defects on glucose, a C_2 independent, glucose-tolerant, and pyruvate-producing evolved Pdc-defiicent *S. cerevisiae* has been reported (van Maris et al., 2004).

Additionally, it is also necessary to intensify the 2,3-BD biosynthetic pathway through introducing the heterologous *alsS* and *alsD* genes from native 2,3-BD forming bacteria and overexpressing the innate *BDH1* gene. As a result, these genes are introduced in the evolved Pdc-deficient strain (SOS4). The resulting strain (BD4) presented the highest yield of 2,3-BD in *S. cerevisiae*, which might be comparable with native 2,3-BD producing bacteria (Alam et al., 1990; Cao et al., 1997; Moes et al., 1985; Petrov and Petrova, 2009, 2010; Sun et al., 2009).

Furthermore, the introduction of an efficient 2,3-BD pathway seemed

to offer the major advantage in terms of reoxidation of cytosolic NADH. Generally, Pdc-deficient strains depend solely on respiration in mitochondria to reoxidate cytosolic NADH generated via glycolysis in the absence of alcoholic fermentation. However, the respiration on glucose is repressed, which leads to the accumulation of cytosolic NADH in cells and thereby retards glucose consumption rate. Introduction of an efficient 2,3-BD pathway might facilitate reoxidation of cytosolic NADH by using NADH as cofactor in converting acetoin to 2,3-BD, which contributed to an improvement of glucose consumption rate. In this Chapter, the BD4 strain harboring the intensified 2,3-BD pathway resulted in a 4.1 fold improvement in glucose consumption rate as compared to the SOS4 strain under oxygen-limited conditions with 20 g/L glucose. Moreover, the BD4 strain was able to utilize 100 g/L glucose completely within 120 h, as opposed to the case where the SOS4 strain only consumed 20 g/L glucose when 100 g/L glucose initially added.

The accumulation of a significant amount of glycerol in the BD4 strain was also observed in parallel with 2,3-BD production under oxygen-limited conditions. It is known that both ethanol and glycerol were produced to maintain redox balance under the limited oxygen condition in *S. cerevisiae* (Bakker et al., 2001; Wang et al., 2001). Glycerol production was demonstrated in the 2,3-BD production by *ADH*

deficient strains (Ng et al., 2012). Therefore, the overproduction of glycerol in the BD4 strain might be thought as an effort to consume surplus NADH after one mole NADH of two moles of cytosolic NADH generated in glycolysis is utilized to produce 2,3-BD.

It is believed that oxygen supply is an important factor affecting product yield, productivity, and by-product formation (Qureshi and Cheryan, 1989; Silveira et al., 1993). High oxygen supply favored cell mass formation, while decreasing the oxygen supply would increase 2,3-BD yield resulting in decreasing the overall conversion rate of cell concentrations (Converti et al., 2003; Sablayrolles and Goma, 1984). Moreover 2,3-BD production from glucose is not redox neutral due to excess NADH. In anaerobic conditions, the stoichiometry for the conversion of glucose to 2,3-BD is given as follows.

2,3-BD production	$C_6H_{12}O_6$	$\rightarrow C_4H_{10}O_2 + 2CO_2 + 2H^+$
Glycerol production	$C_6H_{12}O_6 + 4H^+$	$\rightarrow 2C_3H_8O_3$
	$3C_6H_{12}O_6$	$\rightarrow 2C_4H_{10}O_2 + 2C_3H_8O_3 + 2CO_2$

In this case, the theoretical yield of 2,3-BD and glycerol were 0.333 g 2,3-BD/g glucose and 0.340 g glycerol/g glucose, respectively.

In aerobic conditions, the stoichiometry for the conversion of glucose to 2,3-BD is given in the following equation.

2,3-BD production	$C_6H_{12}O_6$	$\rightarrow C_4H_{10}O_2 + 2CO_2 + 2H^+$
Glycerol production	$C_6H_{12}O_6 + 4H^+$	$\rightarrow 2C_3H_8O_3$
Respiration	$O_2 + 4H^+$	$\rightarrow 2H_2O$
	$5C_6H_{12}O_6 + O_2 \rightarrow 4C_4H_{10}O_2 + 2C_3H_8O_3 + 8CO_2 + 2H_2O$	

In this case, the theoretical yield of 2,3-BD and glycerol were 0.400 g 2,3-BD/g glucose and 0.204 g glycerol/g glucose, respectively. According to supplied oxygen, the BD4 strain can produce 2,3-BD with yield of 2,3-BD ranging from 0.333–0.400 g 2,3-BD/g glucose. Therefore, it is necessary to establish a proper oxygen supplement strategy to ensure efficient 2,3-BD production.

Specially, the BD4 strain did not grow well when aeration was limited suggesting that oxygen supply is a critical factor in the BD4 strain suffering from cofactor imbalance in respect to reoxidation of cytosolic NADH. In the BD4 strain, alleviation of glycerol formation also was observed in aerobic conditions, which suggested that increased respiration by oxygen might contribute to reoxidation of cytosolic NADH instead of formation of glycerol. However, as shown in cultivation with 20 g/L glucose, too much oxygen supply could result in product not 2,3-BD but acetoin as a major product. These results might indicate that the possibility of shortage in NADH which is required to convert acetoin to 2,3-BD due to increased consumption of NADH. In

contrast, 2,3-BD was produced as a major product in cultivation with excess glucose. The BD4 strain produced 34.8 g/L 2,3-BD with the highest yield (0.36 g 2,3-BD/g glucose) and productivity (0.32 g 2,3-BD/L·h) with 1 vvm of aeration and 300 rpm of agitation speed among tested conditions. Aeration is also associated with agitation. The stirring action is particularly important in oxygen supply since it increases the efficiency of fermentation by continuously exposing a new substrate to the culture and disseminating the metabolic end-products throughout the medium (Garg and Jain, 1995; Long and Patrick, 1963). Therefore precise agitation controlling is required for optimization of a fermentation condition.

Although promising results are obtained in this work, it is necessary to improve the productivity as high as that of the 2,3-BD native bacteria by engineering the Pdc-deficient strain in order to enhance glucose consumption and cell growth rate. Moreover, to improve the economics of the fermentation process, it is also desirable to increase 2,3-BD yield by controlling by-product formation through the optimization of fermentative factors including aeration.

Table 4. Strains and plasmids used in Chapter 3

Name	Description	Reference
Strains		
SOS2	D452-2 *Δpdc1*, *Δpdc5* (C_2-dependent and glucose-sensitive)	(Kim et al., 2013a)
SOS4	D452-2 *Δpdc1*, *Δpdc5* (C_2-independent and high glucose-tolerant)	(Kim et al., 2013a)
CON	SOS4 (pRS426GPD, pRS423GPD, and pRS425GPD)	In this Chapter
BD4	SOS4 (pRS426_AlsS, pRS423_AlsD, and pRS425_BDH1)	In this Chapter
Plasmids		
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)
pRS426_AlsS	pRS426GPD harboring *alsS* gene from *Bacillus subtilis* str.168	(Kim et al., 2013a)
pRS423_AlsD	pRS423GPD harboring *alsD* gene from *B. subtilis* str.168	(Kim et al., 2013a)
pRS425_BDH1	pRS425GPD harboring *BDH1* gene from *S. cerevisiae* D452-2	(Kim et al., 2013a)

Table 5. Summary of batch-fermentations by the BD4 strain under different aeration conditions

Aeration condition (aeration / agitation speed)	Concentration of product (g/L)				Yield of 2,3-BD (g 2,3-BD/g Glucose)	Productivity of 2,3-BD (g /L·h)
	DCW	2,3-BD	Acetoin	Glycerol		
0 vvm / 300 rpm	0.8±0.1	6.0±1.2	0.1±0.2	5.0±0.76	0.32 ± 0.05	0.05 ±0.01
0.25 vvm / 300 rpm	10.0±0.7	30.7±0.9	0.8±0.7	15.5±1.8	0.33 ± 0.01	0.26 ± 0.01
1.0 vvm / 300 rpm	13.8±0.1	34.8±0.1	2.8±2.4	5.2±0.6	0.36 ± 0.01	0.32 ± 0.01
1.0 vvm / 500 rpm	14.5±0.3	11.9±1.4	22.7±1.9	0.1±0.1	0.12 ± 0.01	0.14 ± 0.02

Data are averages of two independent experiments.

Table 6. Summary of fed-batch fermentations and cell-recycling fermentation by the BD4 strain

Fermentation / Inoculation OD	Concentration of product (g/L)				Yield of 2,3-BD (g 2,3-BD/g Glucose)	Productivity of 2,3-BD (g /L·h)
	DCW	2,3-BD	Acetoin	Glycerol		
Batch fermentation / OD=1	13.8	34.8	2.8	5.2	0.36	0.32
Fed-batch fermentation / OD=1	12.4	94.5	1.9	27.1	0.30	0.27
Fed-batch fermentation / OD=10	11.0	96.2	3.1	32.1	0.28	0.39
Cell-recycling / OD=60	22.5	81.9	1.0	42.4	0.27	0.88

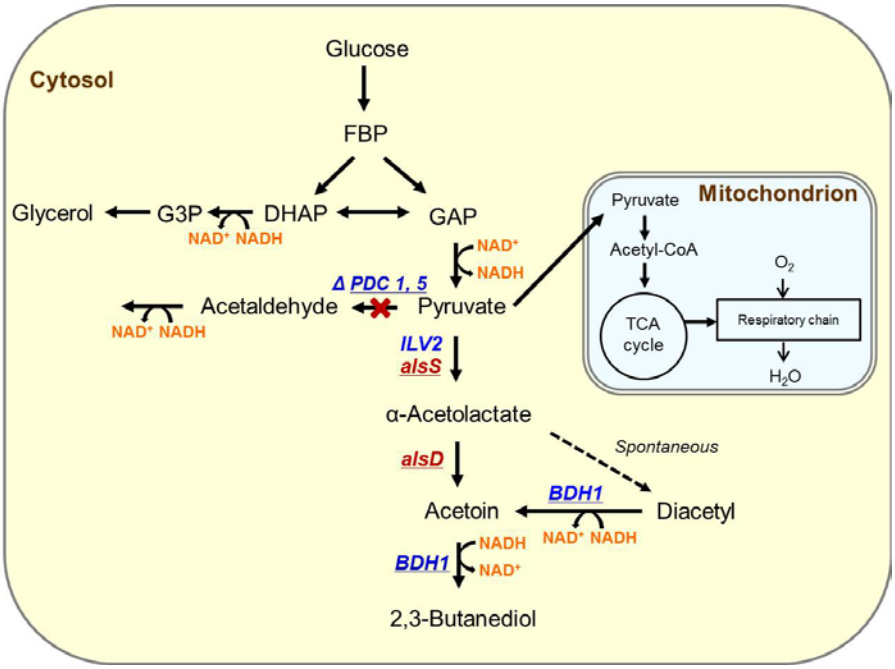


Fig. 8. The metabolic pathway engineering for 2,3-BD production in *S. cerevisiae*. Innate genes are written in blue, while heterologous genes are written in red. Underline means engineered genes.

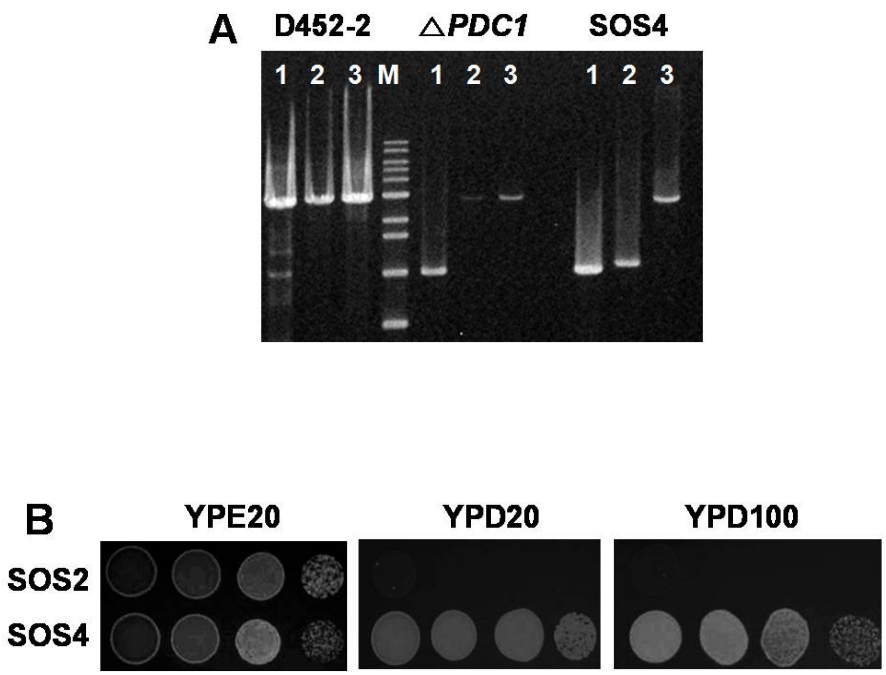
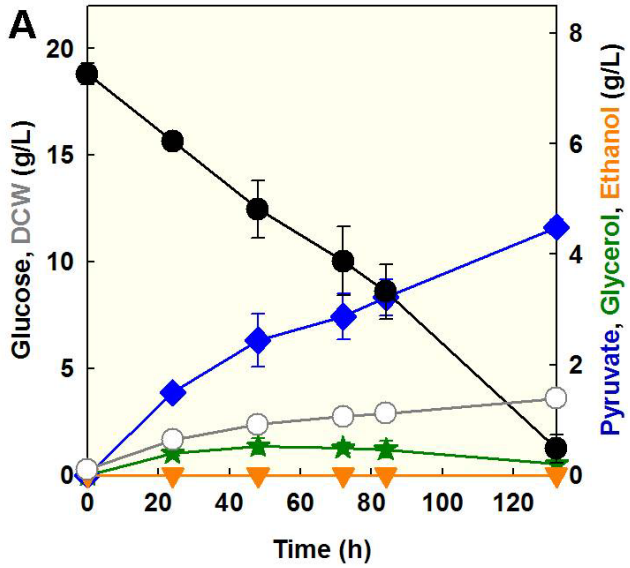
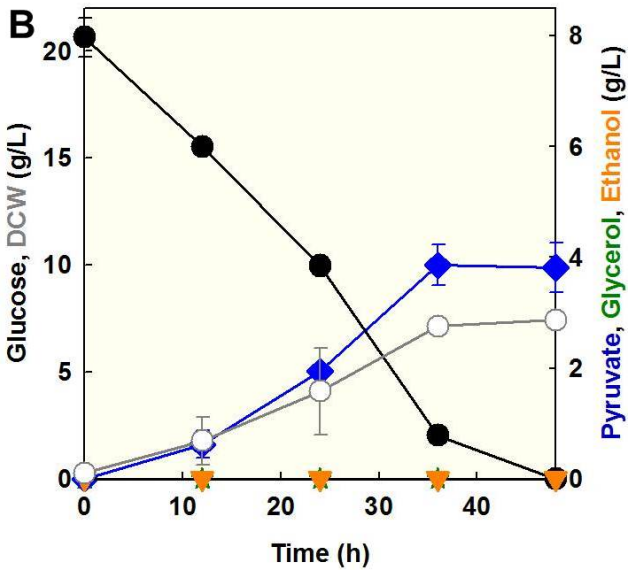


Fig. 9. Confirmation of the Pdc-deficient strain (SOS2) and the evolved Pdc-deficient strain (SOS4).

(A) Diagnostic PCR for confirmation of *PDC1* and *PDC5* deletion. Lane 1: *PDC 1*, Lane 2: *PDC5*, Lane 3: *PDC6*, M: size marker. Deletion of *PDC1* and *PDC5* was confirmed by shorter PCR products from the SOS2 and SOS4 strains.

(B) Growth of the SOS2 strain and the SOS4 strain on YP medium plate with 2% ethanol (YPE20) or 2% glucose (YPD20) or 10% glucose (YPD100).


A
Glucose, DCW (g/L)
Pyruvate, Glycerol, Ethanol (g/L)
Time (h)
0
5
10
15
20
0
2
4
6
8
0
20
40
60
80
100
120


B
Glucose, DCW (g/L)
Pyruvate, Glycerol, Ethanol (g/L)
Time (h)
0
5
10
15
20
0
2
4
6
8
0
10
20
30
40

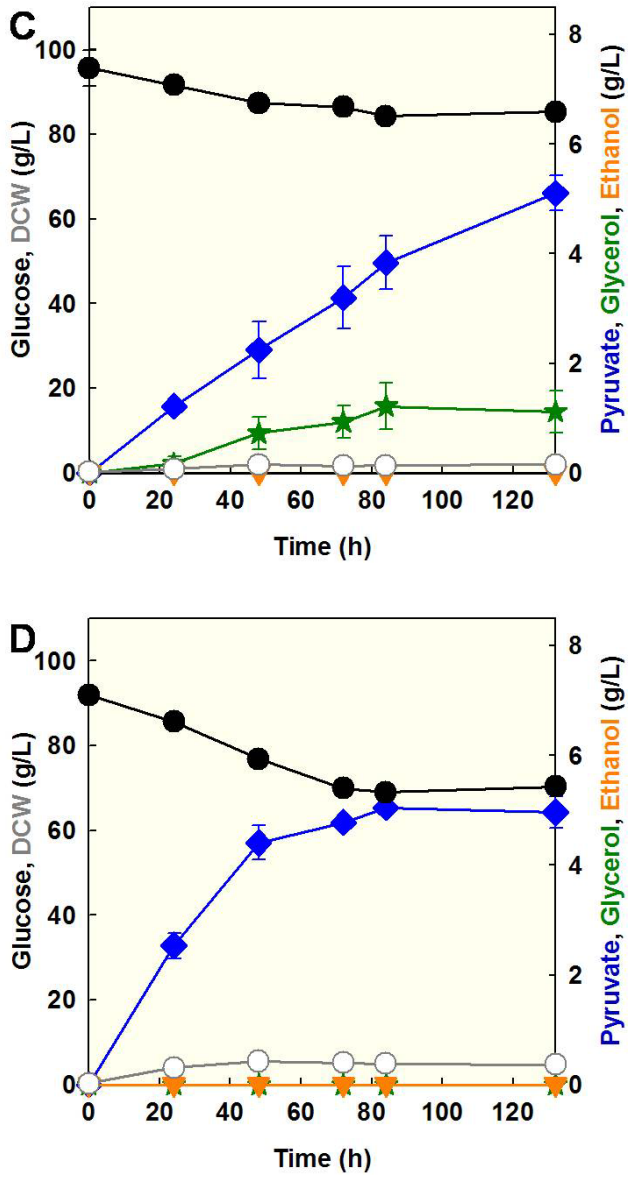
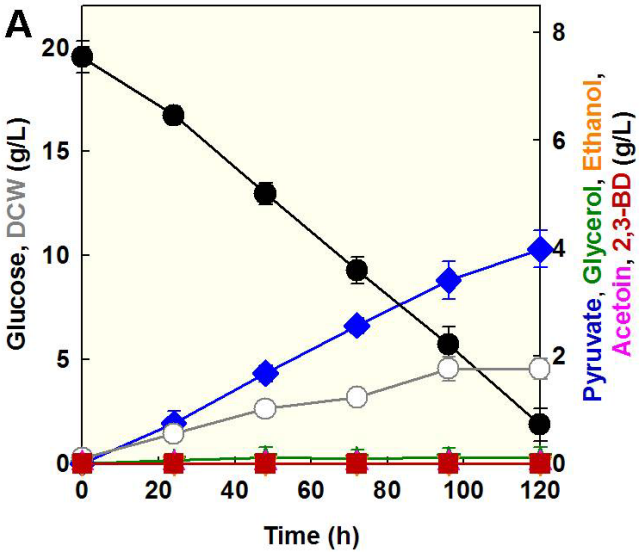
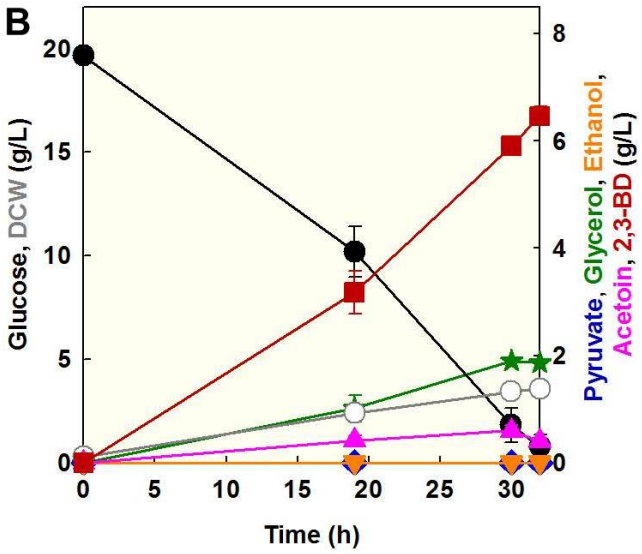


Fig. 10. Batch fermentations by the SOS4 strain in YP medium with glucose as a sole carbon source. (A) and (B): 20 g/L glucose; (C) and (D): 100 g/L glucose; (A) and (C): 80 rpm; (B) and (D): 250 rpm; Symbols: Glucose (●), DCW (○), Pyruvate (◆),Glycerol (★), Ethanol (▼). Error bars represent standard deviations associated with three independent experiments for 80 rpm and with two independent experiments for 250 rpm.


A
Glucose, DCW (g/L)
Pyruvate, Glycerol, Ethanol, Acetoin, 2,3-BD (g/L)
Time (h)
0
5
10
15
20
0
2
4
6
8
0
20
40
60
80
100
120


B
Glucose, DCW (g/L)
Pyruvate, Glycerol, Ethanol, Acetoin, 2,3-BD (g/L)
Time (h)
0
5
10
15
20
0
2
4
6
8
0
5
10
15
20
25
30

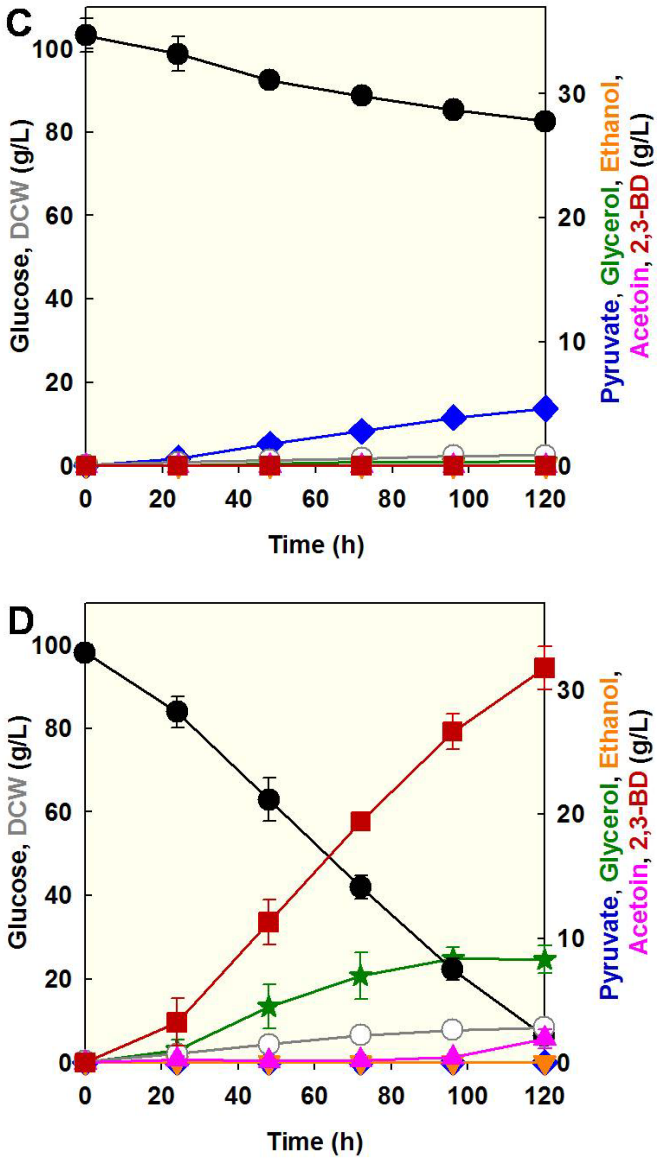


Fig. 11. Comparisons of glucose consumption and 2,3-BD production by BD4 strain under oxygen limited condition. (A) and (C): CON (SOS4 with mother vector); (B) and (D): BD4; (A) and (B): YP medium with 20 g/L glucose; (C) and (D): YP medium with 100 g/L glucose. Symbols: Glucose (●), DCW (○), Pyruvate (◆), Glycerol (★), Ethanol (▼), Acetoin (▲), and 2,3-BD (■). Error bars represent standard deviations associated with three independent experiments. (Except for strain BD4 in 20 g/L glucose, where n=2).

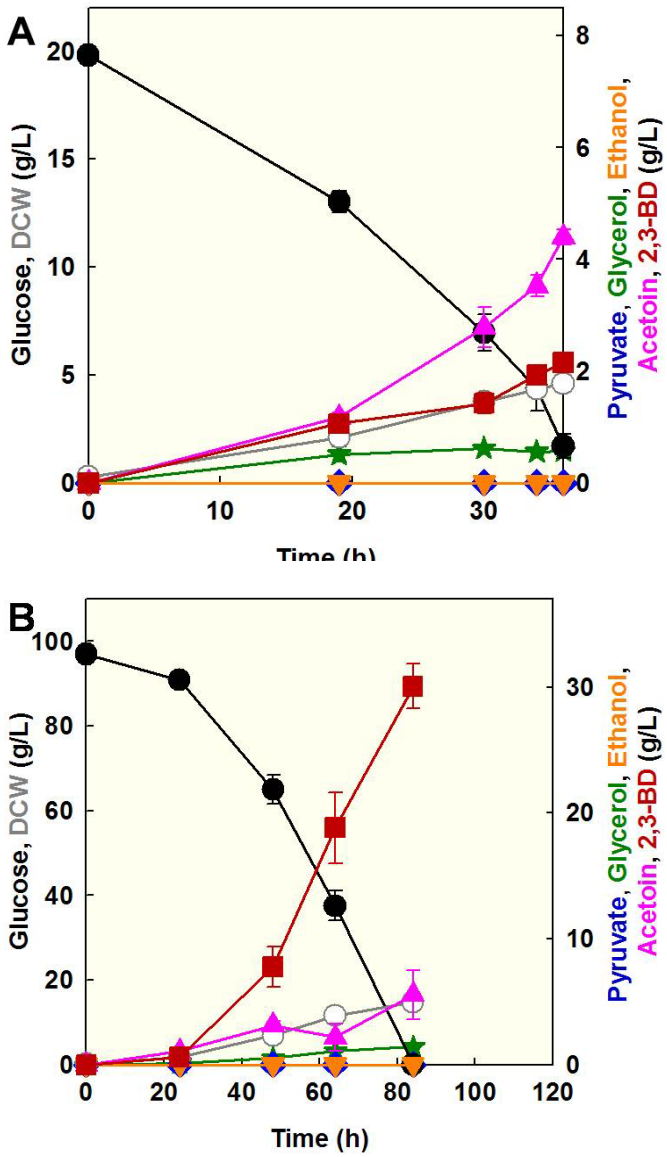
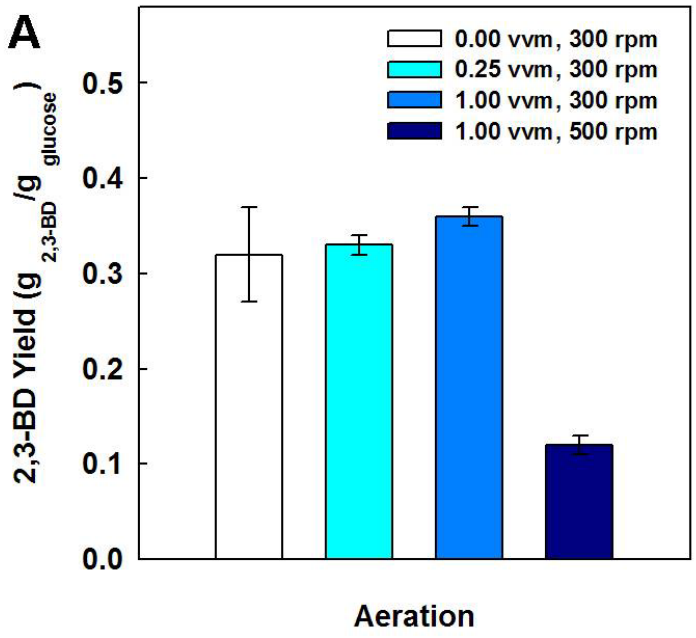
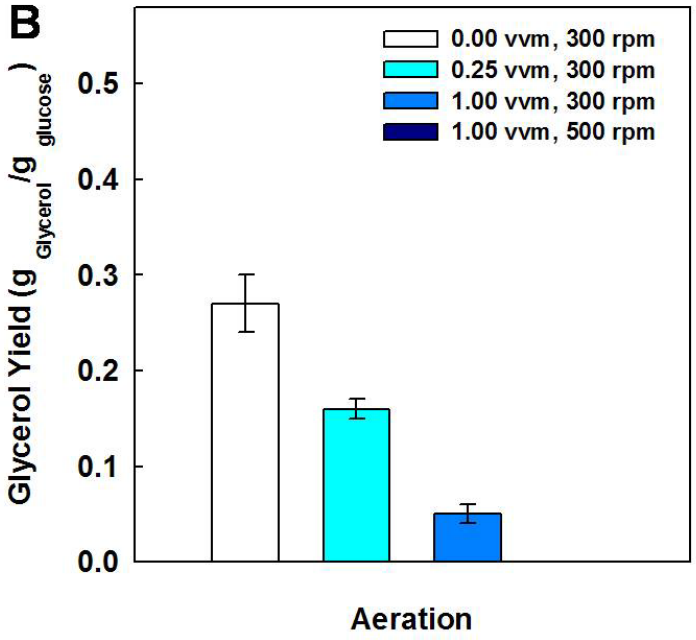


Fig. 12. Comparisons of glucose consumption and 2,3-BD production by the BD4 strain under aerobic condition. (A) YP medium with 20 g/L glucose; (B) YP medium with 100 g/L glucose. Symbols: Glucose (●), DCW (○), Pyruvate (◆), Glycerol (★), Ethanol (▼), Acetoin (▲), and 2,3-BD (■). Error bars represent standard deviations associated with two independent experiments for 20 g/L glucose and with three independent experiments for 100 g/L glucose.


A
0.00 vvm, 300 rpm
0.25 vvm, 300 rpm
1.00 vvm, 300 rpm
1.00 vvm, 500 rpm
2,3-BD Yield (g 2,3-BD /g glucose)
0.0
0.1
0.2
0.3
0.4
0.5
Aeration


B
0.00 vvm, 300 rpm
0.25 vvm, 300 rpm
1.00 vvm, 300 rpm
1.00 vvm, 500 rpm
Glycerol Yield (g Glycerol /g glucose)
0.0
0.1
0.2
0.3
0.4
0.5
Aeration

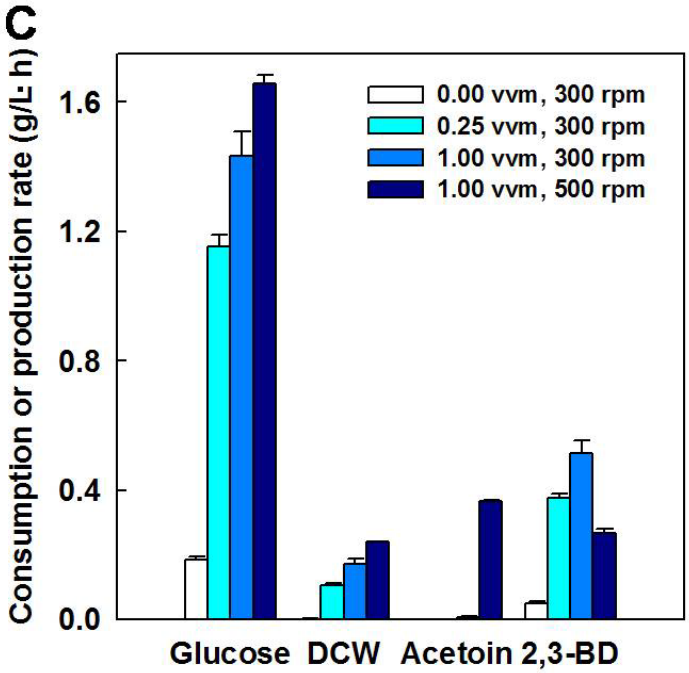
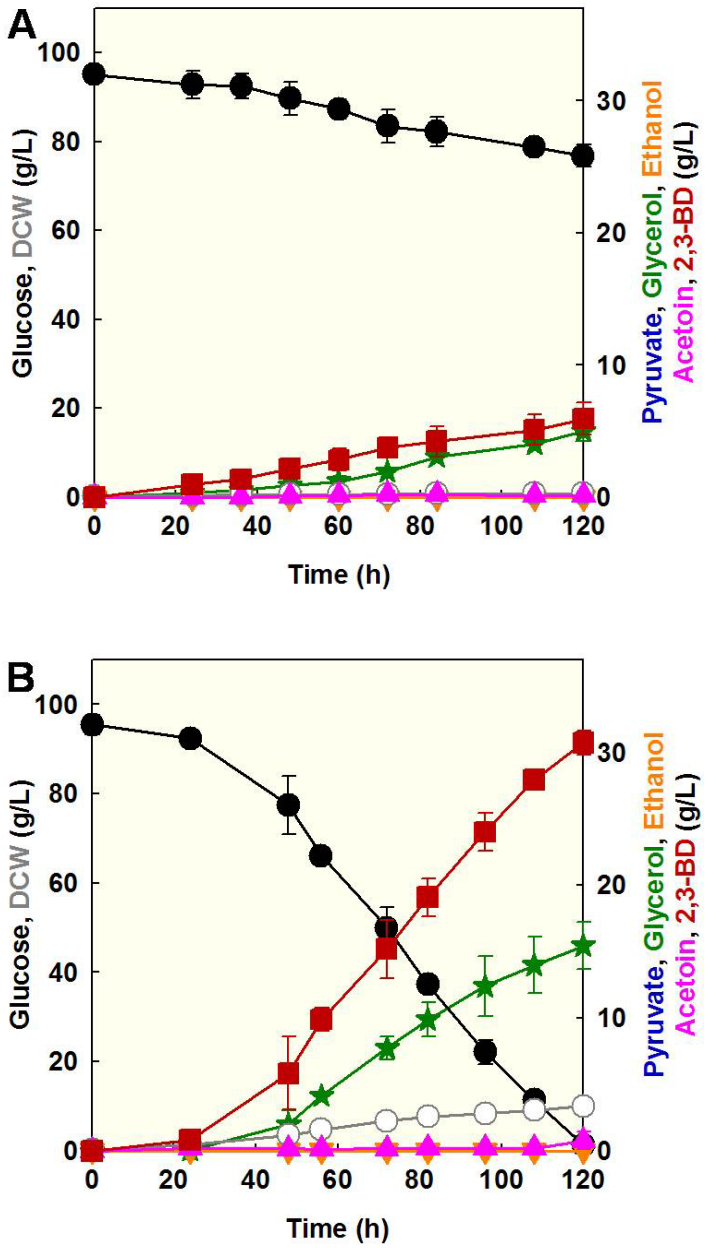


Fig. 13. Effect of oxygen supply on metabolites production in the BD4 strain.

(A) Yield of 2,3-BD; (B) Yield of glycerol; (C) Glucose consumption rate, DCW, acetoin, or 2,3-BD production rate. Symbols: 0 vvm (white bar), 0.25 vvm (sky), 1.0 vvm (blue) with 300 rpm, and 1.0 vvm with 500 rpm (dark blue). Error bars represent standard deviations associated with two independent experiments.


A
Glucose, DCW (g/L)
Pyruvate, Glycerol, Ethanol
Acetoin, 2,3-BD (g/L)
Time (h)
B
Glucose, DCW (g/L)
Pyruvate, Glycerol, Ethanol
Acetoin, 2,3-BD (g/L)
Time (h)

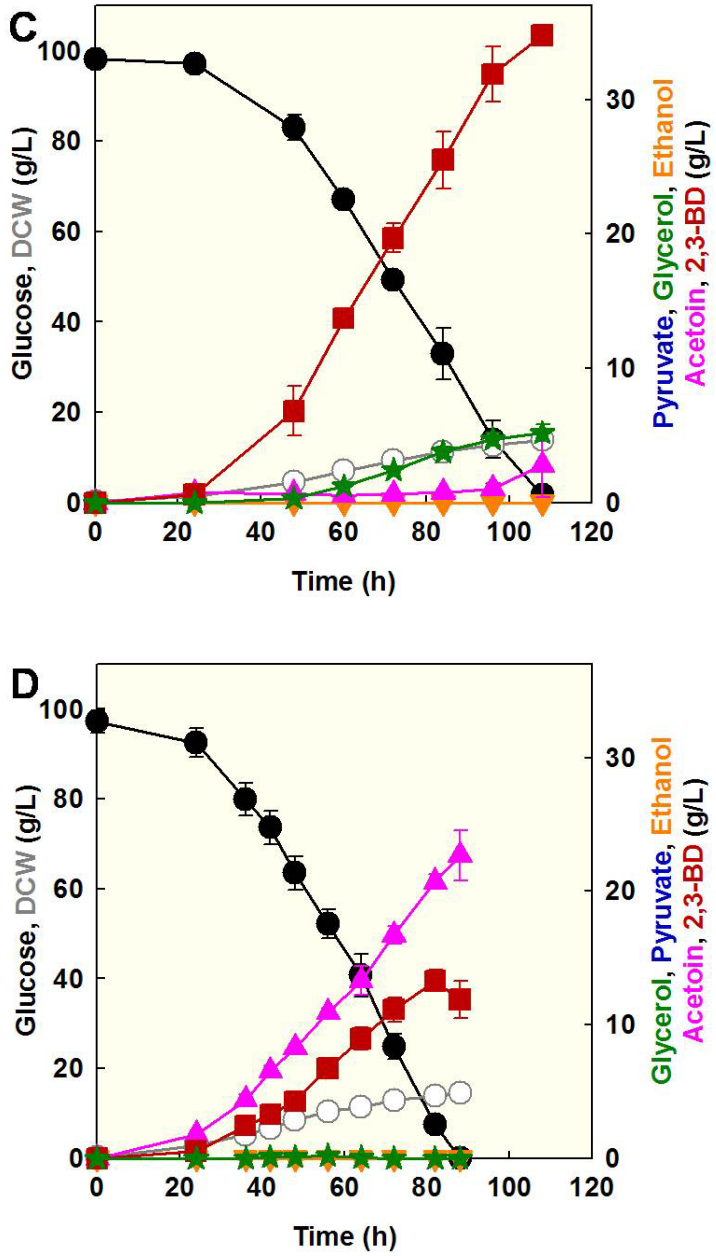


Fig. 14. Batch fermentations by the BD4 strain under different oxygen conditions. (A) 0 vvm, 300 rpm; (B) 0.25 vvm, 300 rpm; (C) 1.0 vvm, 300 rpm; (D) 1.0 vvm, 500 rpm. Symbols: Glucose (●), DCW (○), Pyruvate (◆), Glycerol (★), Ethanol (▼), Acetoin (▲), and 2,3-BD (■). Error bars represent standard deviations associated with two independent experiments.

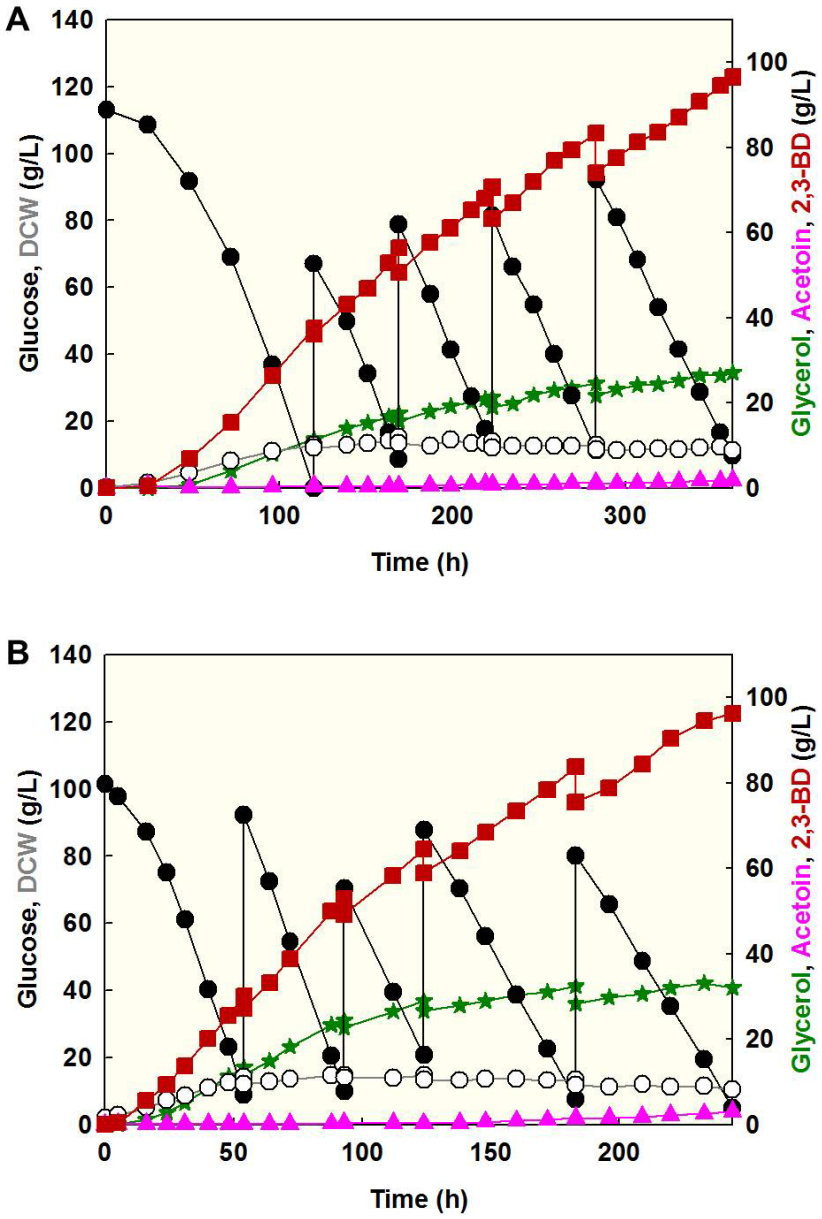


Fig. 15. Fed-batch fermentations by the BD4 strain. (A) Initial OD = 1; (B) Initial OD = 10. Symbols: Glucose (●), DCW (○), Glycerol (★), Acetoin (▲), and 2,3-BD (■).

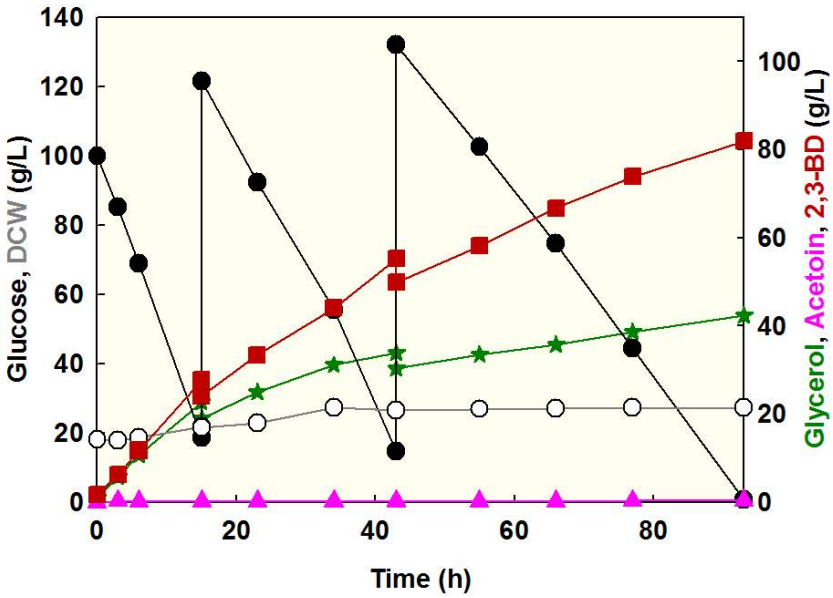


Fig. 16. Cell recycling fed-batch fermentation by the BD4 strain. Symbols: Glucose (●), DCW (○), Glycerol (★), Acetoin (▲), and 2,3-BD (■).

Chapter 4

Production of 2,3-butanediol (2,3-BD) from xylose by pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*

4.1. Summary

While 2,3-butanediol (2,3-BD) can be produced from glucose by natural or engineered microorganisms, 2,3-BD production from xylose that is abundant in lignocellulosic hydrolyzate would make the production of 2,3-BD more sustainable and economical. *Saccharomyces cerevisiae* can produce only trace amounts of 2,3-BD, but also cannot ferment xylose. Therefore, it is necessary to introduce both 2,3-BD production and xylose assimilation pathways into *S. cerevisiae* for producing 2,3-BD from xylose. As ethanol is a major fermentation product by *S. cerevisiae*, a pyruvate decarboxylase (Pdc)-deficient mutant (SOS4) was used as a host in order to increase the carbon flux towards 2,3-BD instead of ethanol. The *XYL1*, *XYL2*, and *XYL3* genes coding for xylose assimilating enzymes derived from *Scheffersomyces stipitis* were introduced into the SOS4 strain to enable xylose utilization. The resulting strain (SOS4X) accumulated pyruvate, a key intermediate for 2,3-BD, from xylose without ethanol production. Additionally, the *alsS* and *alsD* genes from *Bacillus subtilis* and endogenous *BDH1* gene were overexpressed in the SOS4X to increase 2,3-BD production from xylose. As a result, the resulting strain (BD4X) produced 20.7 g/L 2,3-BD with a yield of 0.27 g 2,3-BD/g xylose from xylose. The titer of 2,3-BD from xylose increased up to 43.6 g/L under

a fed-batch fermentation. These results suggest that *S. cerevisiae* might be a promising host for producing 2,3-BD from lignocellulosic biomass for industrial applications.

4.2. Introduction

Recent the rising petroleum prices drew strong interests in microbial production of 2,3-BD production from renewable biomass as an alternative of petroleum-based routes (Li et al., 2010b). In most microbe-based processes, the availability of inexpensive raw materials is a prerequisite to develop an economical fermentation process (Willke and Vorlop, 2004). Among those raw materials, lignocelluose is the most abundant and low-cost biomass on the earth. In microbial 2,3-BD production from lignocellulosic biomass, most studies have been focused on bacterial fermentation using *K. pneumonia*, *K. oxytoca*, and *P. polymyxa*. (Cheng et al., 2010; Gao et al., 2010; Li et al., 2010a; Sun et al., 2009; Wang et al., 2010). Although these bacteria have ability to produce 2,3-BD efficiently from lignocellulosic biomass with a broad substrate spectrum, most microorganisms are classified as potential pathogenic microorganisms. Therefore, industrial-scale fermentation using the 2,3-BD producing pathogenic bacteria might be problematic in the respect of safety and industrialization (Celinska and Grajek, 2009).

S. cerevisiae that offers numerous benefits in terms of safety and large-scale fermentation can be considered as a host for producing 2,3-BD. However, the potential problems related to 2,3-BD production from

lignocellulosic biomass by *S. cerevisiae* exist. First, *S. cerevisiae* is unable to metabolize xylose that is the second most abundant sugar in lignocellulose (Kim et al., 2013b). In order to enable xylose fermentation, heterologous xylose-assimilating pathways have been introduced into *S. cerervisiae* (Ha et al., 2011; Karhumaa et al., 2007; Lee et al., 2012; Madhavan et al., 2009). Among these attempts, introduction of the *XYL1* gene coding for xylose reductase (XR) and *XYL2* gene coding for xylitol dehydrogenase (XDH) from *Scheffersomyces stipites* into *S. cerevisiae* is the most common strategy to engineer xylose-fermenting *S. cerevisiae*. Additionally, the *XKS1* from *S. cerevisiae* or the *XYL3* from *S. stipitis* coding for xylulose kinase (XK) has been introduced to convert efficiently D-xylulose into D-xylulose 5-phosphate, which can be metabolized through the pentose phosphate pathway in yeast (Kim et al., 2013b; Lee et al., 2012). Second, the 2,3-BD yield and productivity by wild type *S. cerevisiae* are too low for industrial productions. While pyruvate is a key precursor for 2,3-BD, it is preferentially used to produce ethanol rather than 2,3-BD. Therefore it is necessary to redirect the carbon fluxes from ethanol to 2,3-BD production. In this Chapter, *S. cerevisiae* capable of producing 2,3-BD from xylose was constructed. Specifically, the xylose assimilation pathway was introduced into a Pdc-deficient strain. The resulting strain accumulated substantial amounts of pyruvate

from xylose, suggesting that it might be a good host for producing 2,3-BD. When a heterologous 2,3-BD biosynthetic pathway was introduced into the pyruvate-accumulating strain. 2,3-BD was produced from xylose very efficiently without production of ethanol (Fig. 17).

4.3. Materials and Methods

4.3.1. Strains and plasmids

Strains and plasmids used in this Chapter are summarized in Table 7. The Pdc-deficient *S. cerevisiae* (SOS4) derived from D452-2 (*MATα*, *leu2*, *his3*, *ura3*, *can1*) (Hosaka et al., 1992) was used for 2,3-BD production from xylose. The SOS4 strain is capable of growing on glucose because of a point mutation in *MTH1* (Kim et al., 2013a). In order to obtain a xylose-fermenting Pdc-deficient strain (SOS4X), XcmI-treated pRS306_XYL123 harboring *XYL1*, *XYL2*, and *XYL3* was integrated into the *URA3* locus of the SOS4 genome. Two plasmids (pRS423_alsS_alsD and pRS425_BDH1) containing the *alsS* and *alsD* genes from *B. subtilis* and the endogenous *BDH1* gene under the control of a constitutive GPD promoter were introduced into the SOS4X strain for 2,3-BD biosynthesis. *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and manipulation.

4.3.2. Medium and culture conditions

E. coli was grown in Lysogeny Broth (LB) medium with 50 μw/mL of ampicillin when required. Yeast strains were cultivated at 30°C in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) with 20 g/L

glucose. To select pre-culture transformants using an amino acid auxotrophic marker, yeast synthetic complete (YSC) medium was used. The YSC medium contained 6.7 g/L yeast nitrogen base, 20 g/L glucose, and appropriate nucleotides and amino acids.

4.3.3. Yeast transformation

Transformations of an integrating cassette for introducing the xylose fermenting pathway and an overexpression cassette for producing 2,3-BD in *S. cerevisiae* were performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). Transformants were selected on YSC medium containing 20 g/L glucose. Amino acids and nucleotides were added as necessary.

4.3.4. Fermentation experiments

Seed cultures were prepared by culturing in 5 mL and 50 mL of YSC medium containing 20 g/L glucose. Yeast cells were then harvested at a mid-exponential phase and inoculated into main cultures with an initial OD600 of ~10. Batch fermentations were performed in 50 mL YP medium containing 4 % of xylose, 8 % of xylose, or a mixture of 2 % glucose and 8 % xylose in a 250 mL flask at 30°C. 80 rpm of agitation speed was set for oxygen-limited conditions. Fed-batch fermentation was performed in 500 mL YP medium with 80 g/L xylose using a 1 L-

bench-top bioreactor (KoBioTech, Korea) under oxygen-limited conditions (0.25 vvm of air flow rate and 300 rpm of agitation speed). During the fed-batch phase, 80 g/L feeding solution composed of 800 g/L xylose was intermittently supplied after depletion of the xylose initially added. 800 g/L glucose solution was also supplied continuously at 1.2 g/h of feeding rate during the growth phase and or 0.3 g/h of feeding rate after growth phase.

Fed-batch fermentations were also performed by intermittently supplying a mixture of glucose and xylose. During the fed-batch phase, 20 g/L glucose and 80 g/L xylose feeding solution composed of 800 g/L xylose or glucose was intermittently supplied after depletion of the sugars initially added. Also, 70 g/L glucose and 40 g/L xylose feeding solution composed of 800 g/L xylose or glucose was supplied after depletion of the sugars initially added during the fed-batch phase.

4.3.5. Analytic methods

Cell growth was monitored by absorbance at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and dry cell weight (DCW) was estimated by conversion of OD600 using a conversion factor: DCW (g/L) = OD600 * 0.3 (Oh et al., 2012). Concentrations of glucose, xylose, pyruvate, xylitol, glycerol, acetate, acetoin, 2,3-butanediol (2,3-BD), and ethanol

were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) with a Bio-Rad Aminex HPX-87H column. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min. Pyruvate was detected by an a UV detector at 214 nm and the others were detected by a refractive index detector.

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

Stereoisomers of 2,3-BD were determined by a gas chromatography (GC) system (Shimadzu GC-2010) equipped with the HP-Chiral-20B GC column (30 m length, 0.32 mm inner diameter) (Agilent). Nitrogen was used as the carrier gas and the flow rate was 30 mL/min. Temperatures of an injector and a flame ionization detector were maintained at 240°C and 250°C, respectively. The column temperature was controlled by the following gradient program: start at 50°C ; increase at a rate of 10°C /min; isotherm at 80°C for 5 min; increase at a rate of 5°C /min; isotherm at 100°C for 7 min; increase at a rate of 40°C /min; isotherm at 240°C for 5 min. A mixture of (*R/S*)-acetoin, (*R*, *R*)-2,3-butanediol, meso-2,3-butanediol, and (*S*, *S*)-2,3-butanediol (Sigma-aldrich) was prepared for a standard of stereoisomers.

4.4. Results

4.4.1. Construction of a xylose-fermenting *S. cerevisiae* accumulating pyruvate (SOS4X)

Even after introduction of the bacterial 2,3-BD biosynthetic pathway into *S. cerevisiae*, the resulting strain produced only trace amounts of 2,3-BD (<1 g/L) from xylose because of unfavorable ethanol production (data not shown) as observed for the conversion of glucose to 2,3-BD in the Chapter 3. In order to redirect the carbon fluxes from ethanol production towards 2,3-BD production, the Pdc-deficient mutant (SOS4) that accumulates pyruvate as a host for producing 2,3-BD was utilized. In order to introduce a xylose metabolic pathway, three genes (*XYL1*, *XYL2*, and *XYL3*) from *S. stipitis* under the control of constitutive promoters were introduced into the Pdc-deficient SOS4 strain via genome integration. To examine growth and accumulation of pyruvate from xylose by the engineered strain (SOS4X), batch-fermentation was performed in YP medium with xylose. When cultured in YP medium with 40 g/L xylose under oxygen-limited conditions, the SOS4X strain consumed only 14 g/L xylose from initial 40 g/L xylose, and accumulated 3.2 g/L pyruvate without production of ethanol after 72 h (Fig. 18). The accumulation of pyruvate from the elimination of ethanol production by the SOS4X strain suggested that the SOS4X

might be a good strain to produce 2,3-BD from xylose.

4.4.2. 2,3-BD production from xylose by the SOS4X strain with the 2,3-BD biosynthetic pathway (BD4X)

As the SOS4X strain was able to consume xylose and accumulate pyruvate instead of ethanol, the 2,3-BD biosynthetic pathway was introduced into the SOS4X strain to convert pyruvate accumulated into 2,3-BD. Specifically, *B. subtilis* genes (*alsS* and *alsD*) coding for α-acetolactate synthase and α-acetolactate decarboxylase, and endogenous *BDH1* gene coding for 2,3-BD dehydrogenase were overexpressed in the SOS4X strain to construct an engineered *S. cerevisiae* (BD4X) capable of producing 2,3-BD from xylose without ethanol production.

As expected, the BD4X strain produced 2,3-BD from xylose without accumulating pyruvate under oxygen-limited conditions. Interestingly, the BD4X strain exhibited faster xylose consumption rate than the control strain harboring control vectors (SOS4X-C). While the SOS4X-C strain accumulated only 3.6 g/L pyruvate in 72 h (Fig. 19A), the BD4X strain produced 8.9 g/L 2,3-BD in 72 h with a faster xylose consumption rate (Fig. 19B). These results suggest that pyruvate generated from xylose in the Pdc-deficient strain (SOS4X) was

converted into 2,3-BD by the 2,3-BD biosynthetic pathway and hence the conversion of pyruvate into 2,3-BD facilitated the xylose consumption rate by the BD4X strain. In order to improve final 2,3-BD titer, a batch fermentation with a higher xylose concentration (80 g/L) was performed under oxygen-limited conditions. As a result, the BD4X strain was able to produce 20.7 g/L 2,3-BD with a yield of 0.27 g 2,3-BD/g xylose after consuming 80 g/L xylose within 114 h. The volumetric productivity of 2,3-BD from xylose was 0.18 g/L·h (Fig. 20A). Additionally, 2,3-BD production by the BD4X strain from a mixture of glucose and xylose (20 g/L glucose and 80 g/L xylose) was examined. The BD4X strain produced 25.8 g/L 2,3-BD with a slightly higher 2,3-BD yield (0.29 g 2,3-BD/g sugar) than that using xylose as a sole carbon source (Fig. 20B). Accumulation of substantial amounts of glycerol was observed in all fermentations by the BD4X strain in parallel with 2,3-BD production.

4.4.3. Characterization of stereoisomer of 2,3-BD produced from sugars by the BD4X strain

To characterize stereoisomer of 2,3-BD produced by the BD4X strain, the supernatants of the cell culture were analyzed by gas chromatography (GC) that can distinguish stereochemistry of various

forms of 2,3-BD. The relative abundances of the stereoisomers of acetoin and 2,3-BD produced by the BD4X strain from glucose or xylose are shown in Fig. 21. According to a previous report, 70–80 % of total 2,3-BD produced by S. *cerevisiae* was (*R*, *R*)-2,3-BD and the remainder was the *meso*-2,3-BD (Herold et al., 1995). Contrasted in the previous study, the BD4X strain accumulated (*R*, *R*)-2,3-BD dominantly (>97 % of the total 2,3-BD) with trace amounts of *meso*-2,3-BD (< 3 %) from glucose or xylose.

4.4.4. Enhanced 2,3-BD production by the BD4X strain under fed-batch fermentation conditions

To determine the potential of the BD4 strain as a 2,3-BD producer from xylose, a fed-batch fermentation experiment was performed with intermittent feeding of xylose. In order to maximize 2,3-BD production from xylose, co-feeding small amounts of glucose from the following reasons was considered. First, glucose metabolism via the pentose phosphate pathway is known to enhance xylose fermentation through replenishing NADPH for the XR reaction (Krahulec et al., 2010). Second, low levels of glucose also contributed to an improvement of xylose uptake whereas high glucose concentrations suppress xylose fermentation (Lee et al., 2000). Third, higher yields of 2,3-BD from a

mixture of glucose and xylose than from xylose was obtained. Therefore a xylose fed-batch fermentation was performed while maintaining low levels of glucose during the fed-batch period. During fed-batch phase, 80 g/L xylose was intermittently supplied after depletion of xylose added. Glucose was constantly fed at the rate of 1.2 g/h during the cell growth phase (0–34 h) and the feeding rate was changed to 0.3 g/h after 34 h. The fed-batch fermentation resulted in 43.6 g/L 2,3-BD after 273 h cultivation with a volumetric productivity of 0.20 g/L·h (Fig. 22). While we achieved the higher titer from the fed-batch fermentation than those from batch fermentations, substantial amounts of glycerol (45.7 g/L) and 4.3 g/L xylitol also accumulated as by-products (Table 8).

The fed-batch fermentations were also carried out with intermittent supply of a mixture of glucose and xylose under two sugar conditions. The BD4X strain produced 43.6 g/L 2,3-BD by intermittent supply of a mixture of 2 % glucose and 8 % xylose (Fig. 23A). In intermittent supply of a mixture of 7 % glucose and 4 % xylose, the BD4X strain produced 49.6 g/L 2,3-BD for 265 h (Fig. 23B) (Table 8).

4.5. Discussion

Both hexose (glucose) and pentose (xylose, arabinose) can be converted into 2,3-BD by several bacteria showing a broad substrate spectrum (Celinska and Grajek, 2009). Among these microbes, *Klebsiella* sp. can produce substantial amounts of 2,3-BD with high yields and productivities from xylose or lignocellulosic hydrolysate (corn hydrolysate, *Jerusalem artichoke* tuber and stalk) (Cheng et al., 2010; Li et al., 2010a; Sun et al., 2009; Wang et al., 2010). Also, the *P. polymyxa* strain could produce 32. 5 g/L 2,3-BD in the pure (*R*, *R*)-form from lignocellulosic biomass (*Jerusalem artichoke* tuber) (Gao et al., 2010). While several studies on bacterial 2,3-BD production from xylose have been reported, there is no attempt to produce 2,3-BD by *S. cerevisiae* from xylose. *S. cerevisiae* is more tolerant to alcohols and inhibitors such as furan derivatives, weak acids, and phenolic compounds present in hydrolysates than other microbes (Olsson and Hahn-Hägerdal, 1993; Park et al., 2011). In this context, *S. cerevisiae* is a desirable microorganism for 2,3-BD production from lignocellulosic hydrolysates. However, wild type *S. cerevisiae* cannot metabolize xylose as a carbon source and exhibits poor 2,3-BD yields due to ethanol production even from glucose. In order to overcome these problems, a Pdc-deficient *S. cerevisiae* capable of fermenting xylose (SOS4X) was constructed for 2,3-BD production. The Pdc-deficient

mutant (SOS4) was constructed by disrupting the *PDC1* and *PDC 5* genes and serial sub-culture on glucose in the previous study (Kim et al., 2013a). Similar to pyruvate production from glucose fermentation by the SOS4 strain in the Chapter 2, the SOS4X strain accumulated pyruvate from xylose without ethanol production under oxygen-limited conditions.

In order to facilitate efficient conversion of pyruvate into 2,3-BD without accumulation of the intermediate metabolites, *B. subtilis* α-acetolactate synthase (AlsS) and α-acetolactate decarboxylase (AlsD), and the endogenous butanediol dehydrogenase (Bdh1) were overexpressed. The resulting strain (BD4X) produced 20.7 g/L 2,3-BD with a yield of 0.27 g 2,3-BD/g xylose from xylose. These titer and yield are be comparable with native 2,3-BD producing bacteria (Jansen et al., 1984; Marwoto et al., 2002).

Introduction of the 2,3-BD biosynthetic pathway into a Pdc-deficient and xylose-fermenting *S. cerevisiae* might be advantageous in the respect to re-oxidation of excess NADH generated from xylose in the absence of ethanol fermentation. The major function of alcoholic fermentation in wild-type *S. cerevisiae* is to re-generate NAD^+ through oxidization of NADH formed in glycolysis. However, excess NADH accumulated in the cytosol from elimination of alcoholic fermentation

in the Pdc-deficient strain might retard cell growth (Ishida et al., 2006). As shown in Fig 19, the BD4X strain exhibited a 2.9-fold improvement in xylose consumption rate (0.53 vs. 0.18 g/L·h) as compared to the control strain (SOS4X-C). This suggests that production of 2,3-BD in the BD4X strain facilitated efficient re-oxidation of cytosolic NADH because the final step of 2,3-BD production from acetoin consumes NADH as a cofactor.

Three stereoisomers of 2,3-BD exist in nature. Optical active [(*R*, *R*)- and (*S*, *S*)] and optical inactive (*meso*-) forms exist. Steroisomeric characteristics of 2,3-BD produced by microbial fermentations are determined by complex interplay of α-acetolactate synthase, acetoin reductase, and 2,3-BD dehydrogenase enzymes. *Klebshiella* sp. and *E. aerogens* produce only *meso*-2,3-BD and (*S*, *S*)-2,3-BD in the proportion of 9:1. *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* produce a mixture of (*R*, *R*)-2,3-BD and *meso*-2,3-BD (Ji et al., 2011). Among the stereoisomers, (*R*, *R*)-2,3-BD is a stereoisomer of interest because it can be utilized for synthesizing specialty chemicals with asymmetric chirality as well as anti-freezing agents with low freezing points (-60°C). (Clendenning, 1946; Marwoto et al., 2004; Soltys et al., 2001). Therefore, production of (*R*, *R*)-2,3-BD with high optical purity is desirable (Xiao et al., 2010). Among the 2,3-BD-producing microbes,

P. polymyxa is known to produce (*R, R*)-2,3-BD with an enantioselective purity of up to 98 % (Li et al., 2013; Yu et al., 2011). In the case of *S. cerevisiae*, it has been reported that 70–80% of 2,3-BD was (*R, R*)-2,3-BD and the remainder was the *meso*-2,3-BD (Fig. 24A) (Gonzalez et al., 2010; Herold et al., 1995; Romano et al., 2003). However it was observed that over 97 % of the 2,3-BD produced by the BD4X strain was (*R, R*)-2,3-BD. In the BD4X strain, acetoin can be only synthesized via α-acetolactate since Pdc activity is removed for the purpose of elimination of ethanol production (Fig. 24B). Also, α-acetolactate decarboxylase (ALDC) was introduced to bypass a reaction via diacetyl, which resulted in production of pure (*R*)-acetoin. Finally, the enantioselective synthesis of pure (*R, R*)-2,3-BD was obtained by overexpression of the *BDH1* gene with strict stereospecificity. Thus, increased (*R*)-acetoin by altering carbon fluxes to the enzymatic decarboxylation pathway might contribute to production of pure (*R, R*)-2,3-BD. This is consistent with the previous research that the engineered *E. coli* has an ability to produce the pure (*R, R*)-2,3-BD through overexpressing the *alsS*, *alsD* genes derived from *B. subtilis* and the genes encoding secondary alcohol dehydrogenases (Yan et al., 2009). Again, this result supports that the BD4X strain might be a promising strain for industrial production.

Despite the encouraging results were obtained in this Chapter, more research would be necessary to further improve 2,3-BD production from xylose by engineered *S. cerevisiae*. First of all, substantial amounts of glycerol simultaneously was accumulated with 2,3-BD production by the BD4X strain. Generally, glycerol production by yeast is driven by NADH oxidization reactions in *S. cerevisiae* (Wang et al., 2001). While two molecules of NAD^+ consumed for generating two molecules of pyruvate in glycolysis, only one molecules of NAD^+ can be re-generated from the reduction of acetoin to 2,3-BD by 2,3-BD dehydrogenase because two pyruvate molecules are condensed to produce one acetoin molecule. Therefore, optimum control of aerations for regenerating NAD^+ is necessary for efficient 2,3-BD production by both native and engineered microorganisms (Kim et al., 2013a; Zeng et al., 1994). Under anaerobic conditions, glycerol production can serve as electron sink as well. In addition to this inherent redox imbalance in 2,3-BD production, the BD4X strain might suffer from surplus NADH generated from xylose metabolism via XR and XDH (Wei et al., 2013). XR prefers NADPH and XDH only use NAD^+ as cofactors so that redox imbalance could aggravate during xylose metabolism (Bruinenberg et al., 1983; Kim et al., 2013b; Kotter and Ciriacy, 1993). This undesirable redox balance might accelerate glycerol production by the BD4X strain (Equation 4.1, 4.2). Thus glycerol production by 2,3-

BD producing *S. cerevisiae* is an evitable phenomenon under oxygen-limited conditions. Glycerol production makes recovering 2,3-BD from fermented broths difficult, which can be obstacle for economical 2,3-BD production. In order to reduce glycerol production, it is required to engineer *S. cerevisiae* through introducing NADH-consuming pathway or leaky ethanol-producing pathway (Wei et al., 2013; Zhang et al., 2012) and to optimize oxygen supplement in fermentation process.

In addition, an improvement in volumetric productivity of 2,3-BD is also needed through co-fermentation of glucose and xylose in respect of sustainable and economical 2,3-BD production from cellulosic hydrolysates.

Equation 4.1. The stoichiometry for the conversion of xylose to 2,3-BD by engineered *S. cerevisiae* harboring NADPH-dependent XR (Fig. 25A)

2,3-BD production $4C_5H_{10}O_5 + O_2 \rightarrow 3C_4H_{10}O_2 + 8CO_2 + 10NADH$

Glycerol production $3C_5H_{10}O_5 + 10NADH \rightarrow 5C_3H_8O_3$

$7C_5H_{10}O_5 + O_2 \rightarrow 3C_4H_{10}O_2 + 5C_3H_8O_3 + 8CO_2$

In this case,

Theoretical yield of 2,3-BD : 0.25 g 2,3-BD/g xylose

Theoretical yield of glycerol : 0.44 g glycerol/g xylose

Equation 4.2. The stoichiometry for the conversion of xylose to 2,3-BD by engineered *S. cerevisiae* harboring NADH-dependent XR (Fig. 25B)

2,3-BD production $6C_5H_{10}O_5 \rightarrow 5C_4H_{10}O_2 + 10CO_2 + 5NADH$

Glycerol production $3C_5H_{10}O_5 + 10NADH \rightarrow 5C_3H_8O_3$

$3C_5H_{10}O_5 \rightarrow 2C_4H_{10}O_2 + C_3H_8O_3 + 4CO_2$

In this case,

Theoretical yield of 2,3-BD : 0.40 g 2,3-BD/g xylose

Theoretical yield of glycerol : 0.20 g 2,3-BD/g xylose

Table 7. Strains and plasmids used in the Chapter 4

Name	Description	Reference
Strains		
SOS4	D452-2 *Δpdc1*, *Δpdc5* (C_2-independent and high glucose-tolerant)	(Kim et al., 2013a)
SOS4X	SOS4 *ura3*::*URA3* pSR6-X123	In this Chapter
SOS4X_CON	SOS4X (pRS423GPD and pRS425GPD)	In this Chapter
BD4X	SOS4X(pRS423_AlsS_AlsD and pRS425_BDH1)	In this Chapter
Plasmids		
pRS306	*URA3*, 2μ origin, Amp^r	
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)
pSR6-X123	*pRS306* $TDH3_P$-*XYL1*-$TDH3_T$, $PGK1_P$-*XYL2*-$PGL1_T$, $TDH3_P$-*XYL3*-$TDH3_T$	(Kim et al., 2012)
pRS423_AlsS_AlsD	pRS423 GPD_P-*alsS*-CYC_T, GPD_P-*alsD*-CYC_T	In this Chapter
pRS425_BDH1	pRS423 GPD_P-*BDH1*-CYC_T	In this Chapter

Table 8. Summary of fed-batch fermentations by the BD4X strain under oxygen-limited condition

	Concentration of product (g/L)					Yield of 2,3-BD (g 2,3-BD/g Glucose)	Productivity of 2,3-BD (g /L·h)
Carbon source	DCW	2,3-BD	Acetoin	Glycerol	Xylitol		
Glucose_limited 80 g/L xylose	7.6	43.6	0.2	45.7	4.4	0.25	0.16
20 g/L glucose 80 g/L xylose	8.3	44.2	0.6	35.4	5.5	0.25	0.17
70 g/L glucose 40 g/L xylose	7.8	49.6	1.0	31.6	3.7	0.26	0.19

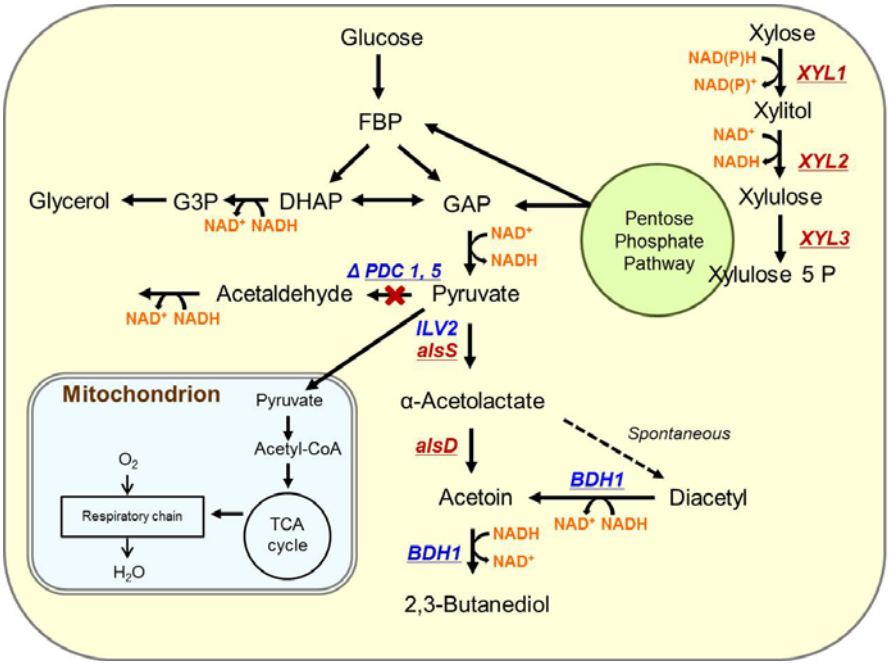


Fig. 17. The metabolic pathway engineering for 2,3-BD production from xylose in *S. cerevisiae.* Innate genes are written in blue, while heterologous genes are written in red. Underline means engineered genes.

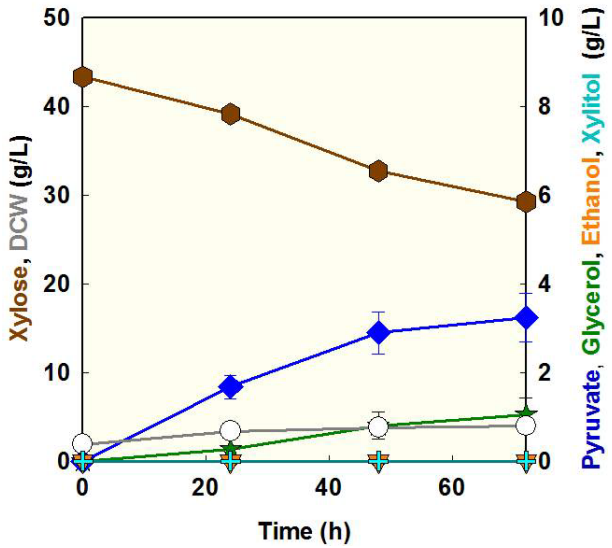


Fig. 18. Batch fermentation by the SOS4X strain in YP medium with 40 g/L xylose as a sole carbon source (YPX40). Symbols: Xylose ( ), DCW ( ), Pyruvate ( ), Xylitol ( ), Glycerol ( ), Ethanol ( ). Error bars represent standard deviations associated with two independent experiments.

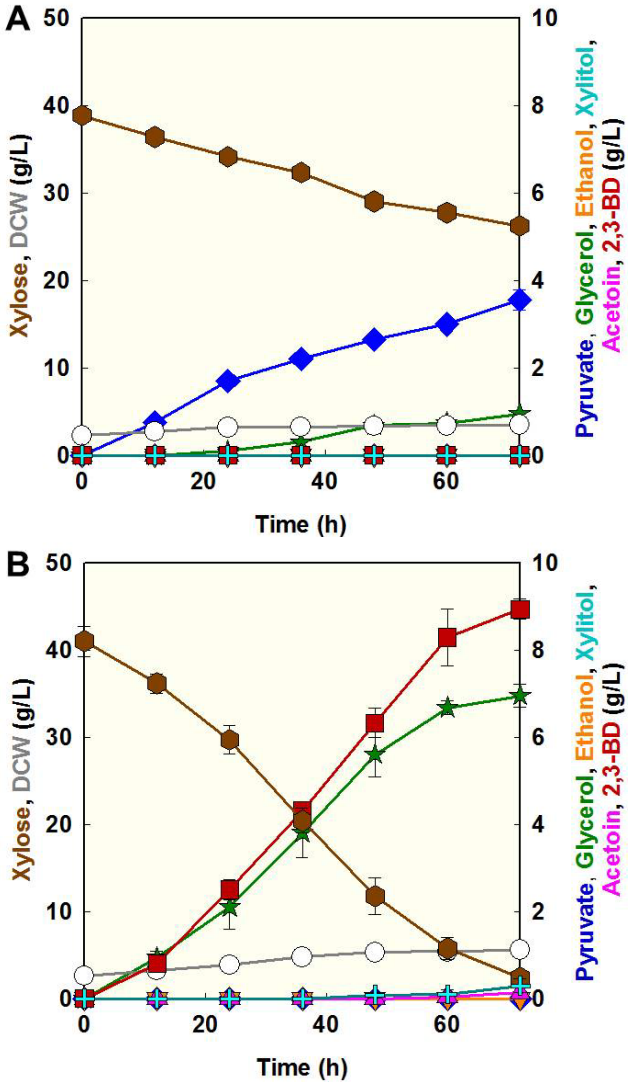


Fig. 19. Comparison of xylose consumption and 2,3-BD production by the BD4X strain. (A) CON in YPX40; (B) BD4X in YPX40. Symbols: Xylose ( ), Glucose ( ), DCW ( ), Pyruvate ( ), Xylitol ( ), Glycerol ( ), Ethanol ( ), Acetoin ( ), and 2,3-BD ( ). Error bars represent standard deviations associated with two independent experiments.

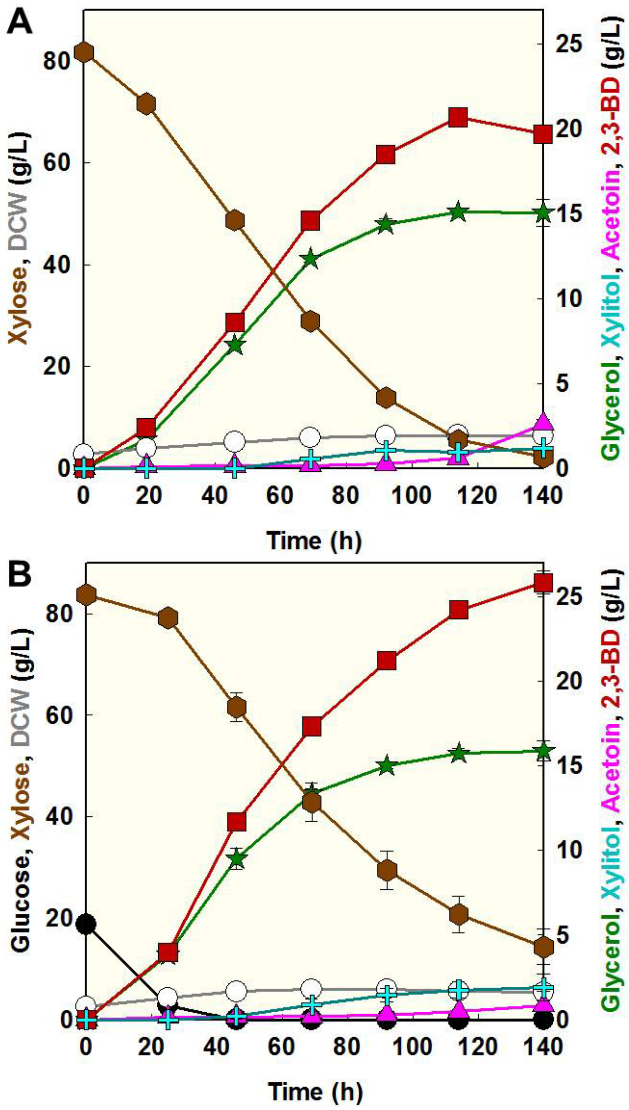


Fig. 20. Batch fermentations by the BD4X strain in YP medium with xylose or with a mixture of xylose and glucose. (A) BD4X in YPX80; (B) BD4X in YPD20X80. Symbols: Xylose ( ), Glucose ( ), DCW ( ), Xylitol ( ), Glycerol ( ), Ethanol ( ), Acetoin ( ), and 2,3-BD ( ). Error bars represent standard deviations associated with two independent experiments.

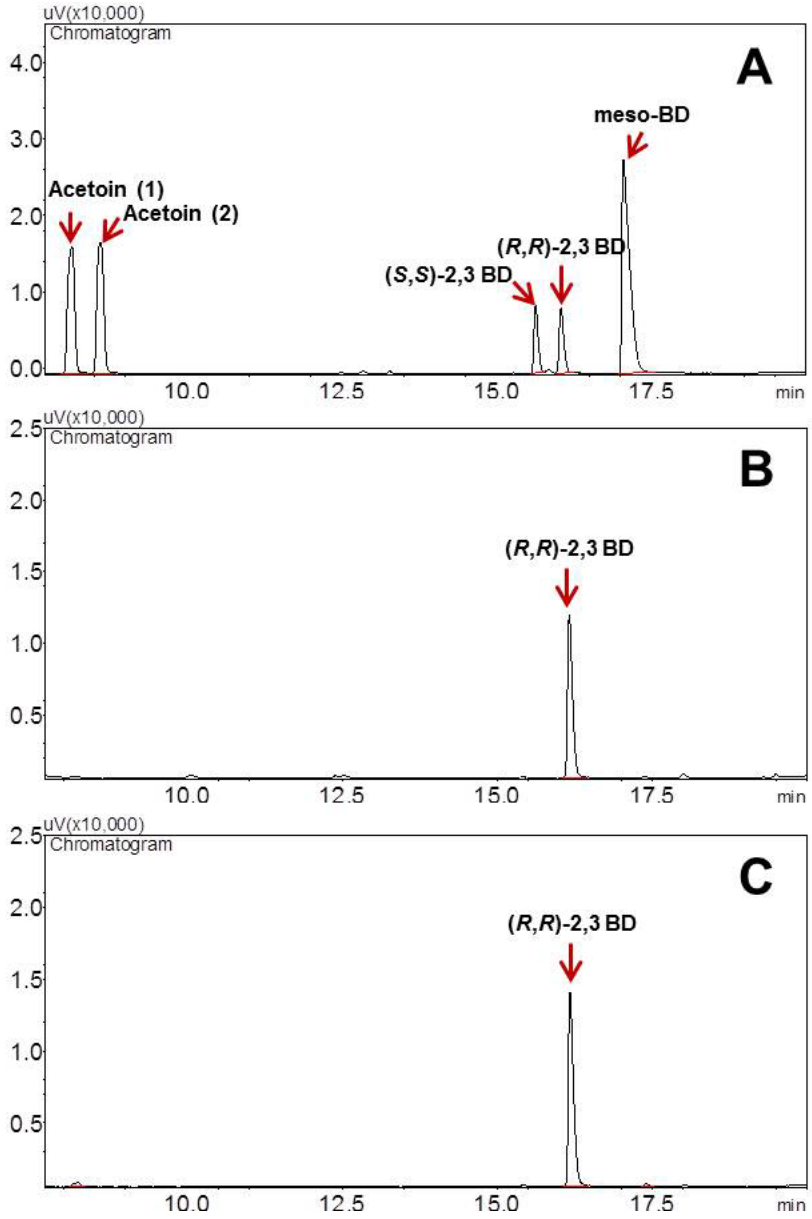


Fig. 21. Gas chromatography analysis of acetoin and 2,3-BD stereoisomers produced by the BD4X. (A) Standard of stereoisomers of acetoin and 2,3-BD; (B) Stereoisomers of acetoin and 2,3-BD produced from glucose; (C) Stereoisomers of acetoin and 2,3-BD produced from xylose.

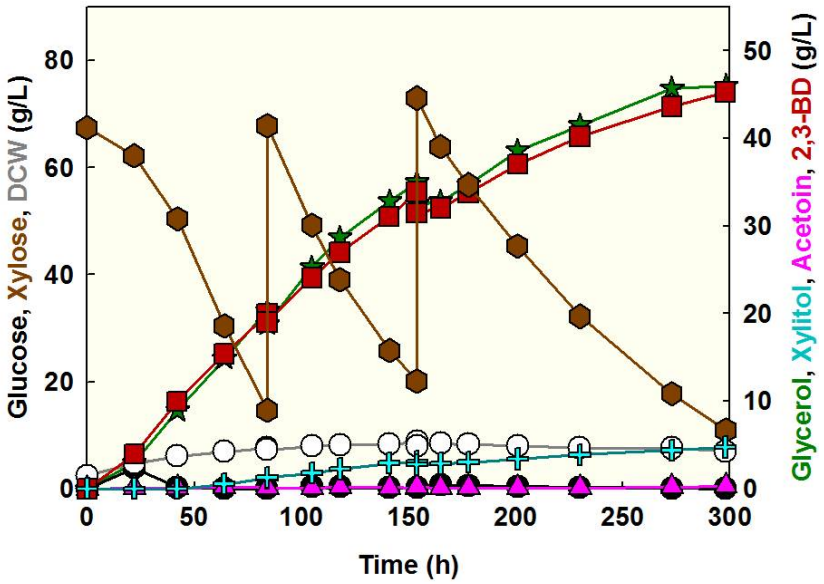


Fig 22. Glucose-limited fed-batch fermentation by the BD4X strain (YPX80_Glucose limited). Symbols: Xylose ( ), Glucose ( ), DCW (open circle), Xylitol ( ), Glycerol ( ), Acetoin ( ), and 2,3-BD ( ).

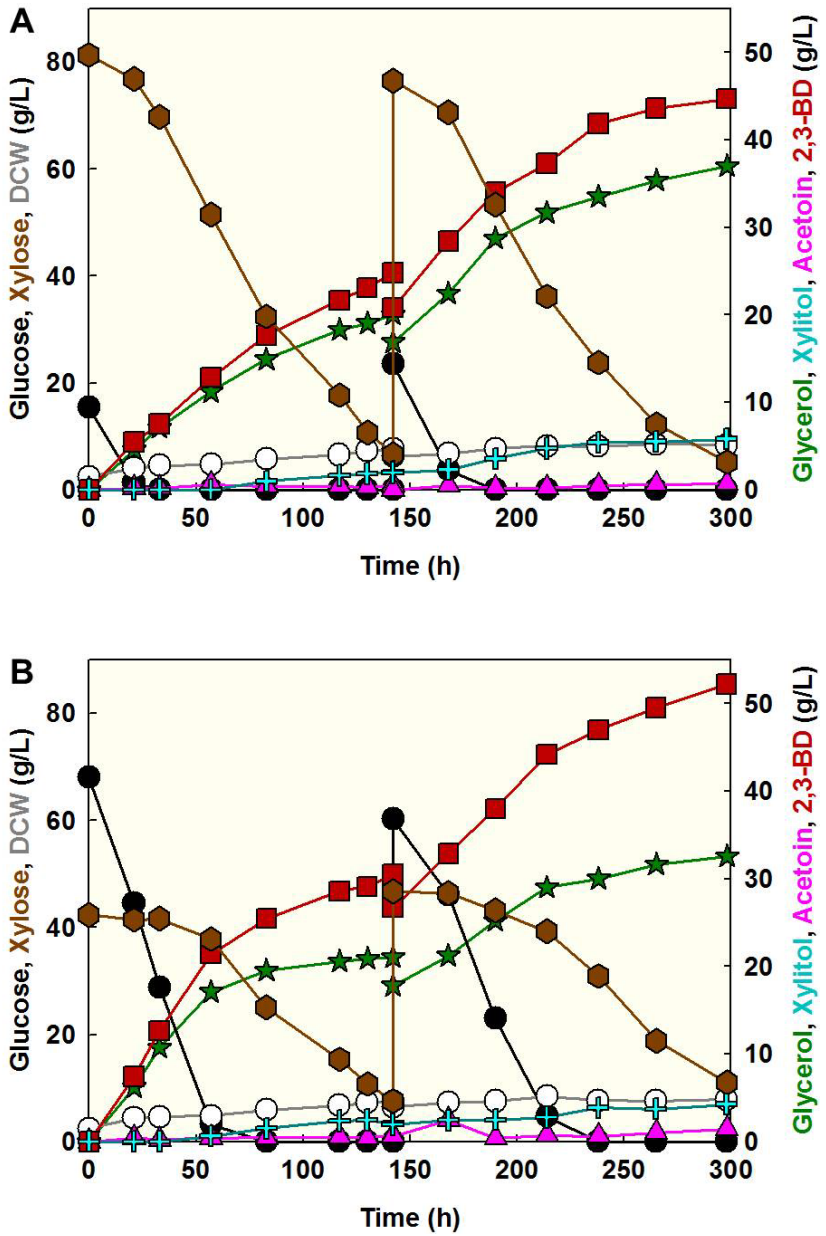


Fig 23. Fed-batch fermentations by the BD4X strain. (A) YPD20X80, (B) YPD70X40. Symbols: Xylose ( ), Glucose ( ), DCW ( ), Xylitol ( ), Glycerol ( ), Acetoin ( ), and 2,3-BD ( ).

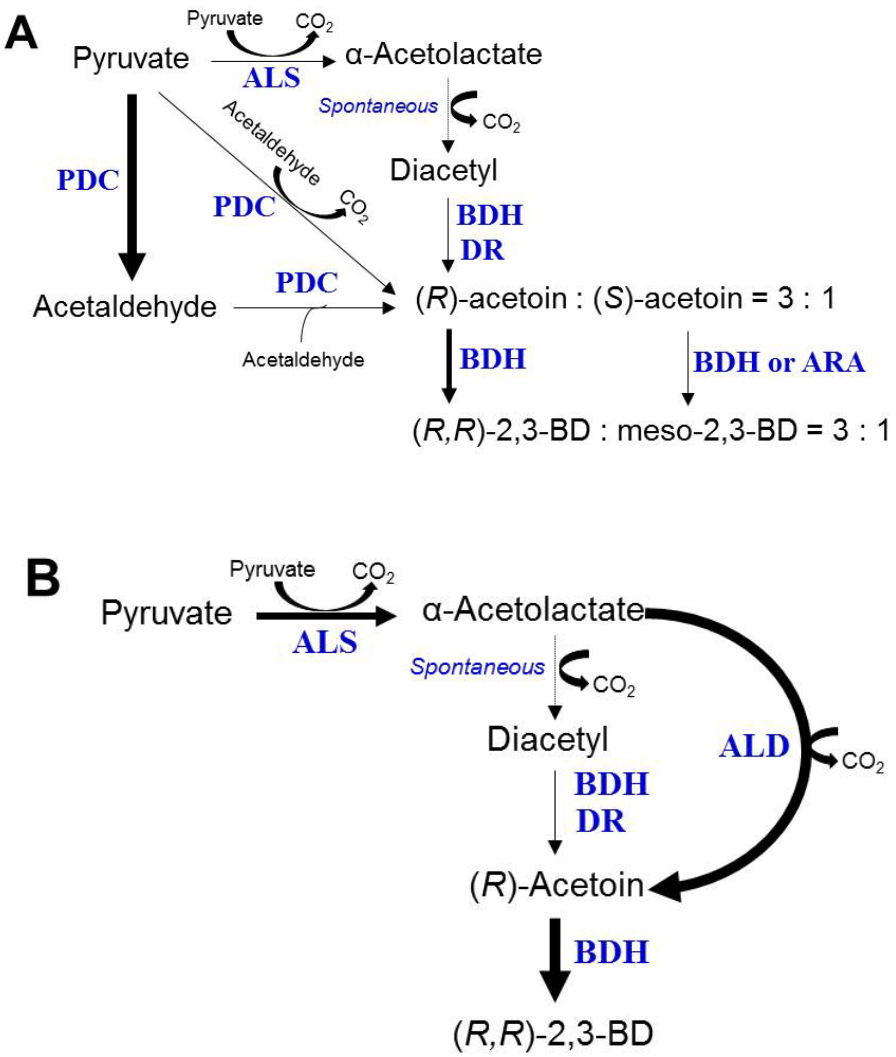


Fig 24. Proposed mechanism for the formation of 2,3-BD stereoisomers in the BD4X strain. (A) Wild-type *S. cerevisiae*; (B) The BD4X strain. PDC: Pyruvate decarboxylase, ALS: α-Acetolactate synthase, BDH: 2,3-butanediol dehydrogenase, DR: Diacetyl reductase, ARA: Arabinose reductase, ALD: α-Acetolactate decarboxylase.

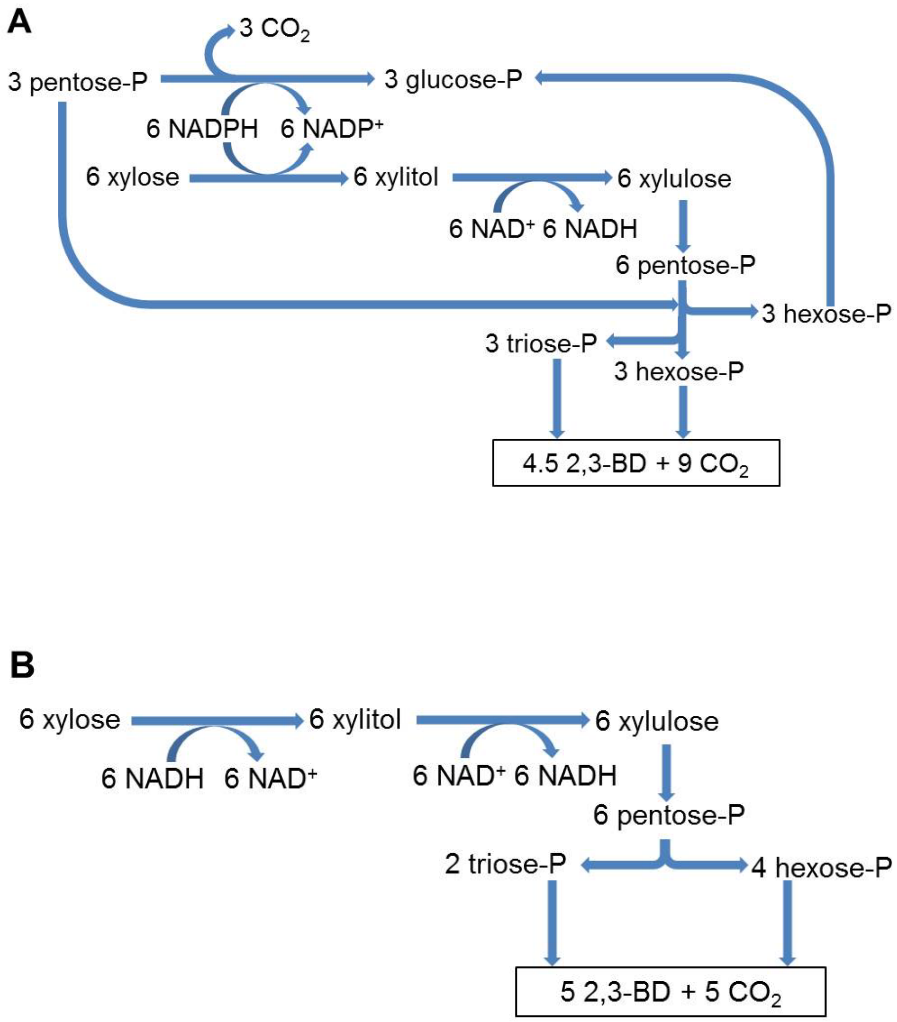


Fig. 25. Schematic representation of xylose metabolism. (A) Xylose metabolism via NADPH-linked xylose reductase, (B) Xylose metabolism via NADH-linked xylose reductase.

Conclusions

This dissertation attempts to produce 2,3-butanediol (2,3-BD) from various carbon sources by engineered *S. cerevisiae*.

2,3-BD is a platform chemical with wide industrial applications. While *S. cerevisiae* that offers numerous benefits in terms of safety and large-scale fermentation can be considered as a host for producing 2,3-BD, there are limiting factors for industrial applications.

Firstly, the 2,3-BD biosynthetic pathway is inefficient as compared to the 2,3-BD producing bacteria. Therefore the key enzymes involved in the 2,3-BD biosynthetic pathway were complemented in order to bypass the inefficient 2,3-BD biosynthetic pathway in *S. cerevisiae* in Chapter 2. The *B. subtilis alsS* gene encoding α-acetolactate synthase and *alsD* gene coding for α-acetolactate decarboxylase, and the innate *BDH1* gene coding for 2,3-butanediol dehydrogenase were overexpressed in *S. cerevisiae*. The engineered 2,3-BD biosynthetic pathway improved 2,3-BD production by a ten-fold compared to the wild strain.

Secondly, the metabolic flux toward 2,3-BD are too low to achieve sufficient 2,3-BD production due to production of ethanol. In Chapter 3, 2,3-BD was produced by using the evoloved Pdc-deficient *S. cerevisiae* (SOS4) without ethanol production. The evolved Pdc-deficient strain harboring the intensified 2,3-BD biosynthetic pathway (BD4)

efficiently produced 2,3-BD as a major product without ethanol production with high yield (0.34 g 2,3-BD/g glucose). In fed-batch fermentation, 2,3-BD concentration increased up to 96.2 g/L.

The final limitation is that *S. cerevisiae* is unable to metabolize xylose, an abundant sugar in lignocellulose. In Chapter 4, an engineered *S. cerevisiae* strain (BD4X) which is able to produce 2,3-BD from xylose was constructed through introducing *XYL1*, *XYL2*, and *XYL3* genes for the xylose assimilating pathway (xylose reductase, xylitol dehydrogenase, and xylulose kinase) and along with the intensified 2,3-BD biosynthetic pathway. The BD4X strain produced 20.7 g/L 2,3-BD in the form of (*R*, *R*)- from xylose with yield of 0.27 g 2,3-BD/g Xylose. In fed-batch fermentation with a mixture of glucose and xylose, the 2,3-BD concentration increased up to 43.6 g/L. These results suggested that *S. cerevisiae* might be a promising host for producing 2,3-BD from lignocellulosic biomass for industrial applications.

Despite the encouraging results were obtained in this study, more research would be required to further improve 2,3-BD production by engineered *S. cerevisiae*. In order to reduce glycerol produced in parallel with 2,3-BD production, efforts are required to alleviate cofactor imbalance in the engineered *S. cerevisiae*. In addition, an improvement in volumetric productivity of 2,3-BD is also needed

through co-fermentation of glucose and xylose in respect of sustainable and economical 2,3-BD production from cellulosic hydrolysates.

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국문 초록

2,3-Butanediol (2,3-BD)는 산업적 활용도가 높은 플랫폼 화학 소재이다. 2,3-BD의 생물공학적 생산에는 주로 박테리아가 사용되는데 고수율로 2,3-BD를 생산할 수 있지만 이들 대부분이 병원성 박테리아로 분류되기 때문에 안전과 산업화 측면에서 대량 생산공정 구축이 어렵다. 그 대안으로 GRAS 미생물로서 안전하다고 알려진 효모를 이용한 2,3-BD 생산에 주목할 필요가 있다. 하지만 효모는 자연상태에서는 2,3-BD를 거의 생산하지 못하기 때문에 고효율의 2,3-BD를 생산하기 위해서는 대사공학적 방법을 이용한 재조합 효모의 구축이 요구된다.

본 연구에서는 효모의 비효율적인 2,3-BD 생합성 경로를 보완하고자 *Bacillus subtilis* 유래의 acetolactate synthase 효소를 암호화하는 *alsS* 유전자와 acetolactate decarboxylase 효소를 암호화하는 *alsD* 유전자를 효모에 도입하였다. 추가적으로 효모 내부의 *BDH1* 유전자를 과발현 시킨 균주를 구축하였다. 그 결과 재조합 BD0 균주에서 생산한 2,3-BD의 농도가 야생 효모보다 10배 이상 증가하였지만 여전히 에탄올이 주된 대사산물로 생성되어 낮은 2,3-BD 수율을 보였다.

따라서 주요 대사 산물인 에탄올 생성을 억제하고자 Pyruvate decarboxylase 활성이 완전히 저해된 균주(SOS4)를 2,3-BD 생산을 위한 모균주로 사용하였다. SOS4 균주의 경우, 에탄올의 생성 없이 2,3-BD의 주요 전구체인 파이루베이트를

축적하였다. 이 SOS4 균주에 2,3-BD 생합성 경로를 강화하기 위하여 *B. subtilis* 유래의 *alsS*와 *alsD* 유전자를 도입하고, 효모 내부의 *BDH1* 유전자를 과발현하여 BD4 균주를 구축하였다. BD4 균주는 2,3-BD를 효과적으로 생산할 뿐만 아니라 포도당 이용 속도가 SOS4 균주에 비해 향상되었다.

BD4 균주를 이용한 2,3-BD 생산은 산소 공급에 영향을 받기 때문에 2,3-BD 생산을 위한 최적의 산소 조건을 탐색하였다. 최적의 산소 공급 조건에서 유가식 배양을 시행한 결과 포도당로부터 2,3-BD를 96.2 g/L 생산할 수 있었다. 이를 통하여 재조합 효모가 2,3-BD의 산업적 생산을 위한 균주로서 가치 있음을 증명하였다.

이와 더불어, 경제적 측면에서 볼 때 목질계 바이오매스로부터 2,3-BD를 생산하는 것은 중요한 일이다. 하지만 효모는 목질계 바이오매스 내에 다량으로 존재하는 자일로스를 대사하지 못하는 단점이 있다. 따라서 이를 개선하고자 *Scheffersomyces stipites* 유래의 자일로스 대사 관련 효소인 xylose reductase, xylitol dehydrogenase, xylulose kinase를 암호화하는 *XR, XDH, XK* 유전자를 SOS4 균주에 도입하였다. 구축된 균주 (SOS4X)는 에탄올 생성없이 자일로스로부터 파이루베이트를 축적하였다.

2,3-BD를 효율적으로 생산하기 위하여 *B. subtilis* 유래의 *alsS, alsD* 유전자를 도입하고 추가적으로 효모 유래의 *BDH1* 유전자를 과발현하여 BD4X 균주를 구축하였다. BD4X 균주는 자일로스로부터 순수한 입체이성체 형태인 (*R, R*)-2,3-BD를 0.25 $g_{2,3\text{-}BD}/g_{Xylose}$ 수율로 생산하였다. 최종적으로 포도당을

제한적으로 공급한 자일로스 유가식 배양에서 총 43.6 g/L의 2,3-BD를 생산하였고, 이는 재조합 효모를 이용한 목질계 바이오매스로부터의 2,3-BD 생산의 가능성을 제시한 점에서 의의를 가진다.

주요어: 2,3-Butanediol (2,3-BD), pyruvate-decarboxylase 결여 효모, 자일로스, 목질계 바이오매스, 유가식 배양

Appendix A:

Deletion of the *PDC1* and *PDC5* genes and laboratory evolution for construction of the evolved Pdc-deficient *S. cerevisiae* (SOS4) (Kim et al., 2013a)

Step 1. The *PDC1* and *PDC5* gene deletion cassette

The method of constructing a gene deletion cassette with homologous regions amplified by PCR reaction was employed for the *PDC1* and *PDC5* gene deletion, and *URA3* marker recycling in this study (Akada et al., 2006). The *PDC* gene deletion cassettes were obtained by PCR using the primers listed in Appendix B. The transformants were selected on YNB URA^- plate and used for diagnostic colony PCR.

Step 2. URA marker recycling

The URA^- transformants, *PDC1* gene deletion strains, were grown in YPD medium at 30°C for 24 h and then directly spread on FOA plates for marker recycling. *PDC5* was sequentially deleted using the same method after isolating a *PDC1* mutant.

Step 3. Laboratory evolution of a Pdc-decifient mutant strain (SOS2)

In order to obtain a C_2-independent and glucose tolerant Pdc-deficient mutant strain, a *PDC1* and *PDC5* double deletion strain (SOS2) was cultured in 50 mL of YP with 1% glucose and 2% acetate, and sub-cultured with gradual reduction of acetate concentrations from 2 to 0% and corresponding increases of glucose concentrations. For adaptation to high-glucose, the resulting C_2-independent culture was transferred to additional serial transfer cultures in 50 mL YP with gradual increases of glucose concentrations from 2 to 10% as a sole carbon source. Growth rates on glucose during the serial transfers on glucose were constantly monitored. Growth rates converged to a maximum when evolved cultures were transferred eight times. After tenth transfers, colonies were isolated from the cultures. A C_2-independent and high-glucose tolerant strain (SOS4) was selected and checked by diagnostic PCR.

Appendix B:

List of primers used in this study

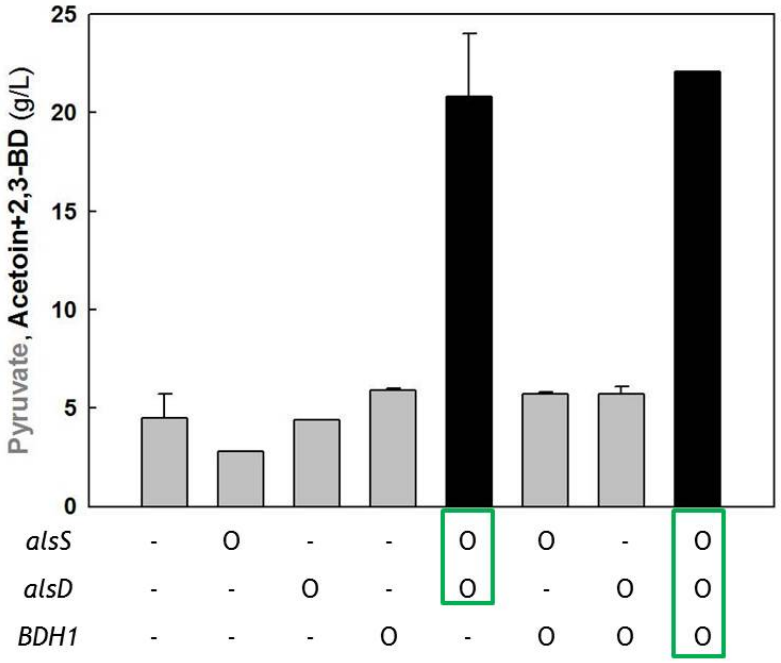
1. List of primers used for construction of the Pdc-deficient strain (SOS2) (Kim et al., 2013a)

Target DNA name (Restriction site)	Sequence (Restriction sites are underlined)
Deletion of *PDC1*	
F-d_PDC1-1 (*Xba*I)	GCTCTAGACTTGAAAAAGGAACAAGCTC
R-d_PDC1-2	GATTTGACTGTGTTATTTTG
F-d_PDC1-3 (*Asc*I)	CAAAATAACACAGTCAAATCGGCGCGCCTTTTTATGTAA CGAAAAATAAATTGGTTCATATTATTACTGATTCGGTAAT CTCCGA
R-d_PDC1-4 (*Xba*I)	GCTCTAGATGCTTATAAAACTTTAACTAATAATTAGAGAT TAAATCGCGGGTAATAACTGATATAATTA
R-d_PDC1-check	GACTGTCGGCAACTTCTTG
Deletion of *PDC5*	
F_d_PDC5-1 (*Xba*I)	GCTCTAGAAGGTTCAAAGACTCTATAAG
R-d_PDC5-2	GTTCTTCTTGTTATTGTATTG
F-d_PDC5-3 (*Asc*I)	CAATACAATAACAAGAAGAACGGCGCGCCTAGTATAATA AATTTCTGATTTGGTTTAAAATATCAACTAGATTCGGTAA TCTCCGAA
R-d_PDC5-4 (*Xba*I)	GCTCTAGA*C*TATATCTATGCCAATTATTTACCTAAACATCT ATAACCTGGGTAATAACTGATATAATTA
R-d_PDC5-check	AGGTACAAAACCGAATACG

2. List of primers used for cloning genes for 2,3-BD biosynthetic enzymes (Kim et al., 2013a)

Target DNA name (Restriction site)	Sequence (Restriction sites are underlined)
alsS from *B. subtilis*	
F_AlsS (*Bam*HI)	CGGGATCCATGTTGACAAAAGCAACAAAAGA
R_AlsS (*Xho*I)	CCGCTCGAGCTAGAGAGCTTTCGTTTTCA
alsD from *B. subtilis*	
F_AlsD (*Bam*HI)	CGGGATCCAAAATGAAACGAGAAAGCAACATTC
R_AlsD (*Xho*I)	CCGCTCGAGTTATTCAGGGCTTCCTTCAG
BDH1 from *S. cerevisiae* D452-2	
F_BDH1 (*Bam*HI)	CGGGATCCAAAATGAGAGCTTTGGCATATTTC
R_BDH1 (*Xho*I)	CCGCTCGAGTTACTTCATTTCACCGTGATTG

Appendix C:



Production of 2,3-BD by the SOS4 strain (evolved Pdc-deficient *S. cerevisiae*) harboring genes involved in the 2,3-BD biosynthetic pathway.

The strains harboring *alsS* and *alsD* genes produced 2,3-BD and acetoin, indicating acetolactate synthase and acetolactate decarboxylase enzymes were expressed as active enzymes in *S. cerevisiae*. Symbol: Pyruvate (grey) and the sum of acetoin and 2,3-BD (black).