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Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*

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HIGHLIGHTS

- A Pdc-deficient *S. cerevisiae* (SOS4) was constructed to produce 2,3-butanediol.
- A point mutation (A81P) in the *MTH1* gene eliciting glucose tolerance was identified.
- A bacterial 2,3-butanediol pathway in the SOS4 enabled 2,3-butanediol production.
- 96.2 g/L of 2,3-butanediol was produced by fed-batch fermentation.

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ABSTRACT

In order to produce 2,3-butanediol (2,3-BD) with a high titer, it is necessary to engineer *Saccharomyces cerevisiae* by deleting the competing pathway and overexpressing the 2,3-BD biosynthetic pathway. A pyruvate decarboxylase (Pdc)-deficient mutant was constructed and evolved for rapid glucose consumption without ethanol production. Genome re-sequencing of the evolved strain (SOS4) revealed a point mutation (A81P) in *MTH1* coding for a transcriptional regulator involved in glucose sensing, unlike the previously reported Pdc-deficient mutant which had internal deletion in *MTH1*. When *alsS* and *alsD* genes from *Bacillus subtilis*, and endogenous *BDH1* gene were overexpressed in SOS4, the resulting strain (BD4) not only produced 2,3-BD efficiently, but also consumed glucose faster than the parental strain. In fed-batch fermentation with optimum aeration, 2,3-BD concentration increased up to 96.2 g/L. These results suggest that *S. cerevisiae* might be a promising host for producing 2,3-BD for industrial applications.

1. Introduction

2,3-Butanediol (2,3-BD) is a multi-functional platform chemical that can be used to produce other bulk chemicals and synthesize diverse products, such as drugs, cosmetics, and industrial solvents (Celinska and Grajek, 2009; Syu, 2001). Especially, dehydration of 2,3-BD into 1,3-butadiene is a promising route to produce a synthetic rubber precursor independent of petroleum (van Haveren et al., 2008; Winfield, 1950).

Microbial production of 2,3-BD from sugar has been reported (Celinska and Grajek, 2009; Ji et al., 2011). Most studies used bacteria, such as *Klebsiella pneumonia*,*Klebsiella oxytoca*, *Enterobacter aerogenes*, and *Paenibacillus polymyxa* to produce 2,3-BD (Cho et al., 2012; Han et al., 2013; Hassler et al., 2012; Jung et al., 2012). While these bacteria are capable of producing 2,3-BD with high yields and productivities, they are classified as pathogenic bacteria so that large-scale fermentation might be difficult in terms of safety and industrialization (Celinska and Grajek, 2009). As an alternative, 2,3-BD production by a GRAS microorganism would be desirable. *Saccharomyces cerevisiae* is an appropriate microorganism in this context. *S. cerevisiae* is known to produce 2,3-BD naturally, but the yield and productivity of 2,3-BD production are poor. Ethanol production is the most obvious barrier for the efficient 2,3-BD production in *S. cerevisiae* because pyruvate, a key intermediate, is preferentially used for producing ethanol rather than 2,3-BD in *S. cerevisiae*. The similar fermentation patterns were observed for production of isobutanol in *S. cerevisiae* (Kondo et al., 2012; Lan and Liao, 2013; Lee et al., 2012; Ofuonye et al., 2013). An inverse relationship between ethanol and 2,3-BD production in *S. cerevisiae* was identified through stoichiometric modeling. Disruption of *ADH1*, *ADH3*, and *ADH5* genes coding for alcohol dehydrogenase achieved decent 2,3-BD titer (2.29 g/L of 2,3-BD) and yield (0.113 g 2,3-BD/g glucose) by engineered *S. cerevisiae* (Ng et al., 2012).

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In order to minimize ethanol production and maximize 2,3-BD production, a pyruvate decarboxylase (Pdc)-deficient mutant can also be utilized for 2,3-BD production. In *S. cerevisiae*, there are three Pdc genes, *PDC1*, *PDC5*, and *PDC6* that contribute to Pdc activity. Among these genes, disruption of *PDC1* and *PDC5* or all three *PDC* genes led to lack Pdc activity (Flikweert et al., 1996; Hohmann, 1991). However, Pdc-deficient strains have potential defects for industrial fermentations. First, 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). Second, the growth of Pdc-deficient strains on fermentable carbon sources such as glucose is also very slow. While respiration is necessary for re-oxidation of cytosolic NADH in Pdc-deficient strains, glucose represses respiration and makes Pdc-deficient strains suffer from redox imbalance (Pronk et al., 1996). However, a C_2-independent and glucose-tolerant Pdc-deficient strain was constructed by laboratory evolution, but the mechanisms underlying the physiological changes were not fully understood despite systems biological endeavor (van Maris et al., 2004). Recently, internal deletion of *MTH1* coding for a negative regulator of glucose-sensing pathway has been identified in the evolved Pdc-deficient strain which is capable of growing on glucose without supplementation of C_2 compounds (Oud et al., 2012). The internal deletion of *MTH1* is likely to cause reduced glucose uptake, which mitigated pyruvate accumulation and redox imbalance from eliminating Pdc activity in the evolved Pdc-deficient strain (Oud et al., 2012).

In 2,3-BD producing bacteria, pyruvate is converted into acetoin via α-acetolactate by acetolactate synthase (ALS), acetolactate decarboxylase (ALDC). Subsequently, acetoin is reduced to 2,3-BD by butanediol dehydrogenase (BDH) (Ji et al., 2011). In *S. cerevisiae*, while pyruvate can be converted into α-acetolactate by Ilv2p involved in the isoleucine and valine synthesis pathway, α-acetolactate cannot be enzymatically decarboxylated into acetoin as acetolactate decarboxylase (ALDC) is not present in *S. cerevisiae*. α-Acetolactate can be converted into diacetyl by spontaneous decarboxylation in the presence of oxygen and then diacetyl can be reduced into acetoin by diacetyl reductase in *S. cerevisiae* (Supplementary Fig. 1). Alternatively, pyruvate decarboxylase is known to condense pyruvate or acetaldehyde into acetoin (Gonzalez et al., 2010). However, metabolic fluxes towards acetoin in *S. cerevisiae* are not high enough to achieve efficient production of 2,3-BD. Therefore, it is necessary to amplify metabolic fluxes in the pathway from pyruvate to acetoin via α-acetolactate. Overexpression of genes (*alsS*, *budA*, and *budC*) coding for enzymes in the bacterial 2,3-BD biosynthesis pathway led to enhanced 2,3-BD production in the wild-type or ADH-deficient *S. cerevisiae* strains (Ng et al., 2012).

In this study, a Pdc-deficient strain was constructed and evolved for growing on glucose. The evolved Pdc-deficient strain was genotyped to identify necessary genetic changes which enable the Pdc-deficient strain to grow on glucose rapidly. Subsequently, the 2,3-BD biosynthetic pathway from *Bacillus subtilis* was introduced into the evolved Pdc-deficient strain to produce 2,3-BD from glucose efficiently in *S. cerevisiae*.

2. Methods

2.1. Strains and plasmids

Strains and plasmids used in this study are described in Table 1. The primers used for construction of Pdc-deficient strains and cloning of genes coding for enzymes in 2,3-BD biosynthetic pathway are listed in Table 2. *S. cerevisiae* D452-2 (*MATα*, *leu2*, *his3*, *ura3*, *can1*) (Hosaka et al., 1992) was used for constructing Pdc-deficient strains and further evolution experiments for isolating a Pdc-deficient mutant (SOS4) which is 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 a constitutive promoter were introduced into the D452-2 and the SOS4 strain. *Escherichia coli* Top 10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and manipulation.

2.2. Medium and culture conditions

E. coli was grown in Lysogeny Broth (LB) medium with 50 μg/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 (YNB), 20 g/L glucose, and appropriate nucleotides and amino acids.

2.3. Yeast transformation

Transformation of knockout and overexpression cassettes for deleting *PDC* genes and introducing 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. For URA marker recycling, Pdc-deficient strains were selected in 5-fluoroorotic acid (FOA) medium. FOA medium

Table 1
Strains and plasmids used in this study.

Name	Description	Reference
Strains		
D452-2	*S. cerevisiae*, *MATα*, *leu2*, *his3*, *ura3*, and *can1*	Hosaka et al. (1992)
SOS2	D452-2*Δpdc1*, *Δpdc5* (C_2-dependent)	In this study
SOS4	D452-2*Δpdc1*, *Δpdc5* (C_2-independent and high glucose-tolerant)	In this study
Wild CON	D452-2 (pRS426GPD, pRS423GPD, and pRS425GPD)	In this study
BD0	D452-2 (pRS426_AlsS, pRS423_AlsD, and pRS425_BDH1)	In this study
CON	SOS4 (pRS426GPD, pRS423GPD, and pRS425GPD)	In this study
BD4	SOS4 (pRS426_AlsS, pRS423_AlsD, and pRS425_BDH1)	In this study
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 *B. subtilis* str.168	In this study
pRS423_AlsD	pRS423GPD harboring *alsD* gene from *B. subtilis* str.168	In this study
pRS425_BDH1	pRS425GPD harboring *BDH1* gene from *S. cerevisiae* D452-2	In this study

Table 2
List of primers used in this study (restriction sites are underlined).

Target DNA name (restriction site)	Sequence
Deletion of *PDC1*	
F-d_PDC1-1 (*Xba*I)	GCTCTAGACTTGAAAAAGGAACAAGCTC
R-d_PDC1-2	GATTTGACTGTGTTATTTTG
F-d_PDC1-3 (*Asc*I)	CAAAATAACACAGTCAAATCGGCGCGCCTTTTTATGTAACGAAAAATAAATTGGTTCATATTATTACTGATTCGGTAATCTCCGA
R-d_PDC1-4 (*Xba*I)	GCTCTAGATGCTTATAAAACTTTAACTAATAATTAGAGATTAAATCGCGGGTAATAACTGATATAATTA
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)	CAATACAATAACAAGAAGAACGGCGCGCCTAGTATAATAAATTTCTGATTTGGTTTAAAATATCAACTAGATTCGGTAATCTCCGAA
R-d_PDC5-4 (*Xba*I)	GCTCTAGACTATATCTATGCCAATTATTTACCTAAACATCTATAACCTGGGTAATAACTGATATAATTA
R-d_PDC5-check	AGGTACAAAACCGAATACG
Cloning of *alsS* from *B. subtilis*	
F_AlsS (*Bam*HI)	CGGGATCCATGTTGACAAAAGCAACAAAAGA
R_AlsS (*Xho*I)	CCGCTCGAGCTAGAGAGCTTTCGTTTTCA
Cloning of *alsD* from *B. subtilis*	
F_AlsD (*Bam*HI)	CGGGATCCAAAATGAAACGAGAAAGCAACATTC
R_AlsD (*Xho*I)	CCGCTCGAGTTATTCAGGGCTTCCTTCAG
Cloning of *BDH1* from *S. cerevisiae* D452-2	
F_BDH1 (*Bam*HI)	CGGGATCCAAAATGAGAGCTTTGGCATATTTC
R_BDH1 (*Xho*I)	CCGCTCGAGTTACTTCATTTCACCGTGATTG

was prepared according to published procedures (Akada et al., 2006).

2.4. Deletion of the PDC1 and PDC5 genes and laboratory evolution

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 (Supplementary Fig. 2) (Akada et al., 2006). The *PDC* gene deletion cassettes were obtained by PCR using the primers listed in Table 2. The transformants were selected on YNB URA^- plate and used for diagnostic colony PCR. 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.

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 8 times. After 10 transfers, colonies were isolated from the cultures. A C_2-independent and high-glucose tolerant strain (SOS4) was selected and checked by diagnostic PCR (Supplementary Fig. 3).

2.5. Fermentation experiments

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

Bioreactor fermentations were performed in 500 mL YP medium with 100 g/L glucose using a 1 L-bench-top fermentor (KoBioTech, Korea) at 30 °C and pH 5.5. To provide various levels of oxygen transfer, aeration was controlled with combinations of different air flow rates (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 fermentor. Yeast cells were harvested at mid-exponential phase and inoculated into bioreactor with initial OD_{600} of ~10. The initial glucose concentration was 100 g/L of glucose. During the repeated batch fermentation, 100 g/L of feeding solution composed of 800 g/L of glucose was continuously supplied when glucose was depleted. 300 rpm of agitation and 1.0 vvm of aeration were maintained throughout the cultivation.

2.6. 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 UV detector at 214 nm and the others were detected by refractive index detector. All reagents including (2R,3R)-2,3-BD for standard solution were purchased from Sigma–Aldrich.

2.7. Genome sequencing

Genomic DNA from the *S. cerevisiae* D452-2 and the Pdc-deficient strain SOS4 were prepared by Genomic DNA preparation kit (Zymo Research). The shotgun DNAseq libraries were prepared with TruSeq DNAseq Sample Prep kit (Illumina). Average DNA fragment size was 660 base pairs (bp), ranging from 530 to 930 bp. The

libraries were quantitated by qPCR and sequenced on one lane for 101 cycles from each end of the fragments on a HiSeq2000 using a TruSeq SBS sequencing kit version 3 and processed with Casava 1.8.2 in the Biotechnology Center in University of Illinois, Urbana-Champaign. The sequence reads were trimmed of low-quality data with a quality score limit of 0.05 and adaptor sequence in CLC Genomics Workbench 6.0.2 (CLC Bio, Aarhus, Denmark, http://www.clcbio.com) and reads of less than 15 bp in length were discarded. Trimmed short-read sequences were assembled using reference-guided assembly (map reads to reference) against a published *S. cerevisiae* S288c genome sequence in CLC Genomics Workbench. Probabilistic variants were detected with the following factors: minimum coverage = 10 and probability = 90 and a filter ignoring non-specific matches and broken pairs. The variants were filtered against reference variants from *S. cerevisiae*. Annotation and amino acid change analysis were carried out with the following short-read parameters: mismatch cost = 2, insertion cost = 3, deletion cost = 3. Match mode was random to allow for assembly of both inverted repeat regions and repetitive elements.

3. Results and discussion

3.1. Introduction of a 2,3-BD biosynthetic pathway into S. cerevisiae

While 2,3-BD can be synthesized from pyruvate via intermediate compounds such as α-acetolactate, diacetyl, and acetoin in *S. cerevisiae*, the slow oxidative decarboxylation involved in conversion of α-acetolactate to acetoin via diacetyl (Dulieu and Poncelet, 1999; Yamano et al., 1994) might be regarded as a limiting step to produce 2,3-BD in *S. cerevisiae*. To overcome the problem, *alsS* and *alsD* genes coding for acetolactate synthase and acetolactate decarboxylase from *B. subtilis* were overexpressed. Additionally, the endogenous *BDH1* gene coding for 2,3-butanediol dehydrogenase (Bdh1) was overexpressed for reducing acetoin into 2,3-BD. In order to compare 2,3-BD production between the resulting strain (BD0) and the control strain (Wild CON) harboring control vectors, batch fermentations were performed in YP medium containing 20 g/L of glucose under oxygen-limited conditions. The Wild CON strain produced 7.6 g/L of ethanol as a major product and trace amounts (<0.1 g/L) of 2,3-BD, whereas the BD0 strain produced 0.7 g/L of 2,3-BD with reduced ethanol production (7.0 g/L) (Supplementary Fig. 4A and B). However, the BD0 strain still produced unfavorable ethanol as a major product, suggesting that elimination of ethanol production is necessary for efficient 2,3-BD production.

3.2. Construction of non-ethanol producing and pyruvate accumulating S. cerevisiae by deletion of both PDC1 and PDC5 genes

As our initial attempts to produce 2,3-BD in *S. cerevisiae* through introduction of the bacterial 2,3-BD biosynthetic pathway resulted in trace amounts (<1 g/L) of 2,3-BD production because of ethanol production, a mutant *S. cerevisiae* which cannot produce ethanol but does accumulate pyruvate was constructed. The resulting mutant was used as a host strain for introducing the bacterial 2,3-BD biosynthetic pathway. Both *PDC1* and *PDC5* genes coding for pyruvate decarboxylase (Pdc) were deleted to generate a Pdc-deficient mutant. The resulting Pdc-deficient strain (SOS2) was not able to produce ethanol, but grew on glucose medium only when C_2 compound such as acetate or ethanol was supplemented as previously reported (Flikweert et al., 1996; van Maris et al., 2004). In order to overcome the growth defect on glucose, laboratory evolution based on serial subcultures was performed to isolate a C_2-independent and high glucose-tolerant Pdc-deficient *S. cerevisiae* (SOS4). As described in Supplementary Fig. 3B, the SOS2 strain was not able to grow in YP with either 20 g/L or 100 g/L of glucose, but the SOS4 strain grew well in YP media with both 20 g/L and 100 g/L of glucose. Growth and accumulation of pyruvate by the SOS4 strain on glucose were examined. When cultured in YP medium with 20 g/L of glucose as a sole carbon source, the SOS4 strain consumed 20 g/L of glucose within 120 h under oxygen-limited conditions, not producing ethanol but accumulating 4.5 g/L of pyruvate (Fig. 1a). Under aerobic conditions, the SOS4 strain also accumulated 3.8 g/L of pyruvate and showed faster rates of glucose consumption and growth as compared to the oxygen-limited conditions (Fig. 1b). The accumulation of pyruvate and elimination of ethanol production in the SOS4 strain during glucose fermentation indicates that Pdc activity in the SOS4 was almost completely eliminated. However, the SOS4 strain was not able to utilize all the supplied glucose in the medium. Only 15 or 20 g/L of glucose was utilized from the initial 100 g/L of glucose under oxygen-limited or aerobic conditions, respectively (Fig. 1c and d).

3.3. Genotyping the C_2-independent and glucose tolerant Pdc-deficient strain (SOS4)

Next generation genome sequencing of the SOS4 strain was performed to identify genetic changes responsible for the C_2-independent and improved glucose tolerant phenotypes. The genomic sequences of the mother strain D452-2 and the evolved strain SOS4 were compared. A single nucleotide polymorphism (SNP) located at the 241 bp (from G to C) position of the *MTH1* gene in the SOS4 strain was found and confirmed by Sanger sequencing of a PCR product containing the *MTH1* gene amplified from genomic DNA of the SOS4 strain (Fig. 2). The SNP leads to an amino acid change from alanine to proline at the 81 codon in Mth1 which is a transcriptional regulator involved in glucose sensing. Recently, the Mth1 mutant capable of suppressing the growth defect of Pdc-deficient strains on glucose has been reported (Oud et al., 2012). An evolved Pdc-deficient strain (TAM) exhibiting tolerance to glucose was found to harbor a mutant *MTH1* (*MTH1-ΔT*) which has a 225 bp internal deletion from position 169 to 393 bp (Fig. 2). Compared to the wild-type of *MTH1* in the unevolved Pdc-deficient strain (RWB837), the *MTH1-ΔT* mutant allele in the TAM strain lost its phosphorylation site (Moriya and Johnston, 2004) and PEST regions (Rechsteiner and Rogers, 1996) which are important for degradation of Mth1. As a result, the internal deletion might elicit glucose tolerance phenotype in the TAM strain via reduced degradation of Mth1. The point mutation found in this study at position 241 bp of *MTH1* from the evolved Pdc-deficient strain (SOS4) is not located in the phosphorylation site and PEST regions, but the point mutation is located in the internal deletion site (169–393 bp) found in the previous study (Oud et al., 2012) (Fig. 2). The point mutation (A81P) might lead to a protein structure change which delays proteolytic degradation of Mth1 with a similar mechanism whereby the internal deletion (*MTH1-ΔT*) elicits glucose tolerance phenotype in a Pdc-deficient *S. cerevisiae*.

Several *MTH1* mutants exhibiting improved glucose tolerance from glucose-sensitive parental strains have been reported (Lafuente et al., 2000; Schulte et al., 2000). A suppressing mechanism by mutant Mth1s against the growth defect of the Pdc-deficient strains or glucose intolerance can be postulated from a function of Mth1 for regulation of the *HXT* genes coding for hexose transporters. Without extracellular glucose, Mth1 represses transcription of the *HXT* genes with the transcriptional regulator 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 enhanced glucose uptake rate (Kim, 2009; Lafuente et al., 2000; Moriya

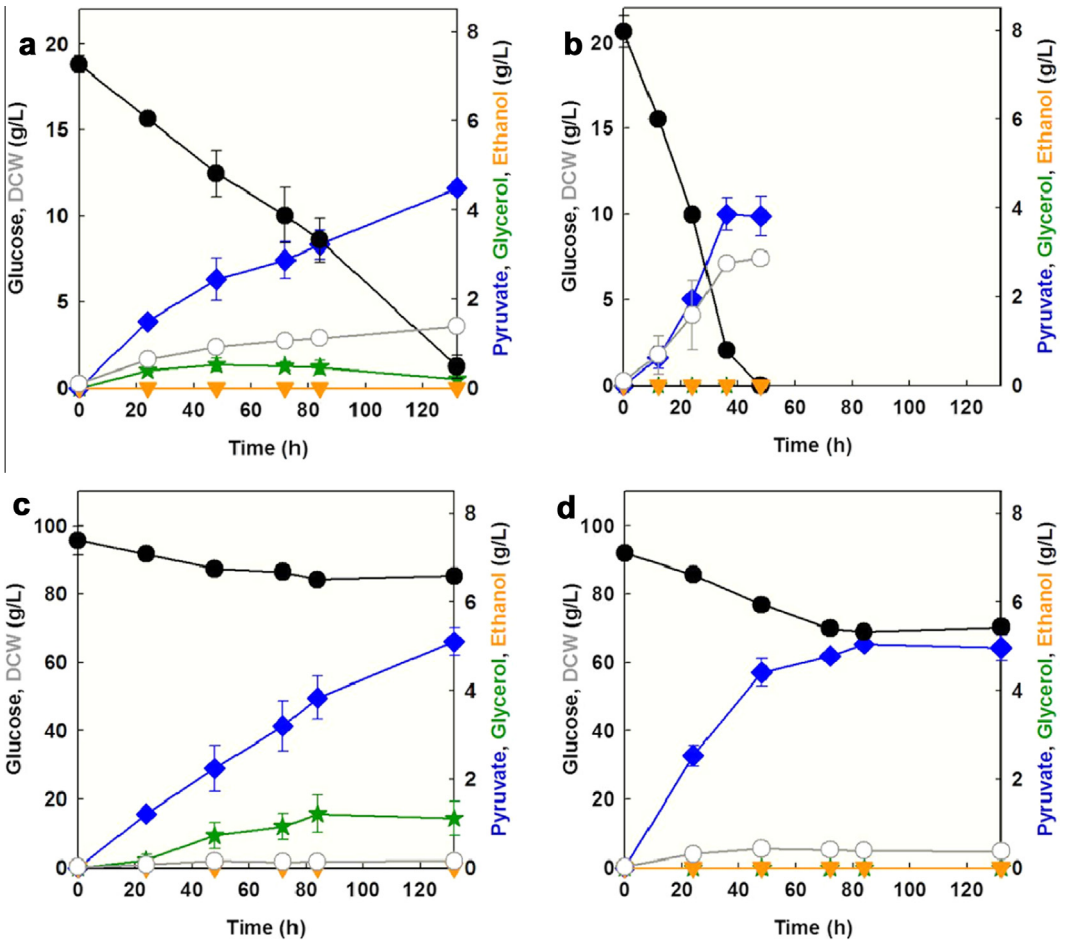

Fig. 1. Fermentation profiles by the SOS4 strain in a complex medium using glucose as a sole carbon source under different aeration conditions. (a) YP medium with 20 g/L of glucose at 80 rpm; (b) YP medium with 20 g/L of glucose at 250 rpm; (c) YP medium with 100 g/L of glucose at 80 rpm; (d) YP medium with 100 g/L of glucose at 250 rpm. Symbols: Glucose (circle), DCW (open circle), Pyruvate (diamond), Glycerol (star), and Ethanol (triangle down). Error bars represent standard deviations associated with three independent experiments for 80 rpm and with two independent experiments for 250 rpm.

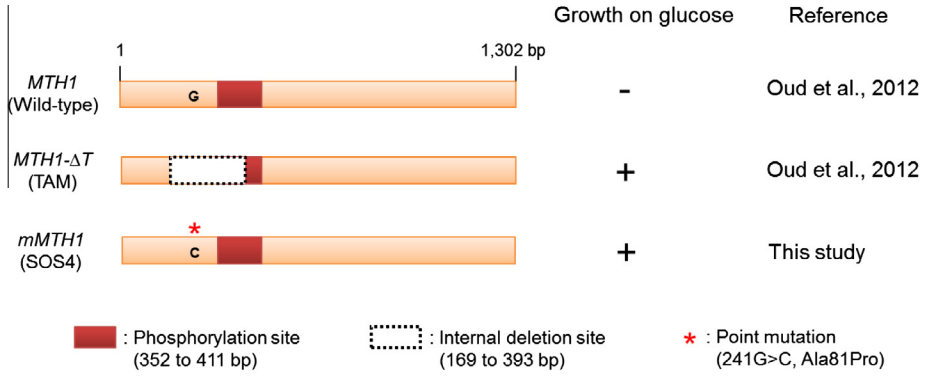

Fig. 2. Comparison of the isolated mutant allele of *MTH1* from two Pdc-deficient mutants (TAM and SOS4) exhibiting glucose tolerance. The evolved Pdc$^-$ strain TAM has the 225 bp internal deletion site from 169 to 393 bp in the *MTH1-ΔT* allele which is missing a phosphorylation site and putative PEST regions. The evolved Pdc-deficient strain SOS4 has a point mutation from G to C at the 241 bp of *MTH1* gene (*mMTH1*) leading to an amino acid change from alanine to proline (Ala81Pro).

and Johnston, 2004). However, mutant Mth1s, which are inefficiently phosphorylated or not phosphorylated at all, might 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 slow and fixed glucose uptake rate by the Mth1 mutants might be responsible for suppression of the growth defect of Pdc-deficient strains on glucose, alleviating intracellular accumulation of pyruvate and redox imbalance caused from elimination of metabolic fluxes towards ethanol production.

3.4. Production of 2,3-BD by a Pdc-deficient S. cerevisiae through introducing a 2,3-BD biosynthetic pathway

As the isolated SOS4 strain did not produce ethanol and accumulated pyruvate, the 2,3-BD biosynthetic pathway was intro-

duced into the SOS4 strain for converting pyruvate into 2,3-BD. As expected, the resulting strain (BD4) produced 2,3-BD without ethanol production, and the BD4 strain 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 of pyruvate in 120 h (Fig. 3a), the BD4 strain produced 6.4 g/L of 2,3-BD in 32 h with rapid glucose consumption (Fig. 3b). These results suggest that accumulated pyruvate in the Pdc-deficient strain (SOS4) can be converted into 2,3-BD by the 2,3-BD biosynthetic pathway showing 4.1-fold improvement in glucose consumption rate as compared to the control strain.

In order to improve final 2,3-BD titer, a batch fermentation with 100 g/L of glucose was also carried out under oxygen-limited conditions. The CON strain utilized only 20 g/L of supplied glucose accumulating 4.6 g/L of pyruvate (Fig. 3c). However, the BD4 strain was able to consume 100 g/L of glucose within 120 h and produced 31.8 g/L of 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. 3d). Introduction of the efficient 2,3-BD biosynthetic pathway seemed to offer the major advantage in terms of reoxidation of cytosolic NADH. Generally, Pdc-deficient strains depend solely on respiration in the mitochondria to re-oxidize cytosolic NADH generated via glycolysis in the absence of alcoholic fermentation (Pronk et al., 1996). However, the respiration on glucose medium is repressed, which leads to accumulation of cytosolic NADH in cells and thereby retards cell growth and glucose consumption rate (Ishida et al., 2006). Introduction of the efficient 2,3-BD biosynthetic pathway might facilitate reoxidation of cytosolic NADH by using NADH as cofactor in converting acetoin to 2,3-BD, which contributed to enhancing glucose consumption rate.

Accumulation of significant amounts of glycerol was also observed, in parallel with 2,3-BD production, by the BD4 strain under oxygen-limited conditions. *S. cerevisiae* produces ethanol and glycerol with coupled regeneration of NAD^+ to maintain redox balance in the cytosol under oxygen-limited conditions (Bakker et al., 2001; Wang et al., 2001). Since ethanol production is eliminated in BD4, production of 2,3-BD and glycerol with coupled regeneration of NAD^+ is necessary to maintain redox balance. Therefore, glycerol production is likely to be inevitable when producing 2,3-BD in *S. cerevisiae* under oxygen-limited conditions. Enhanced glycerol production was also observed in ADH-deficient strains for 2,3-BD production (Ng et al., 2012).

2,3-BD production by the BD4 strain was examined under aerobic conditions by increasing agitation speed up to 250 rpm. The BD4 strain produced substantial amounts (4.3 g/L) of acetoin instead of 2,3-BD and accumulated less glycerol when 20 g/L of glucose was used under aerobic conditions (Supplementary Fig. 5A). However, the product formation pattern can be altered by increasing the initial glucose concentration. When 100 g/L of glucose was supplied, the BD4 strain produced 2,3-BD as a major product and small amounts of acetoin even under aerobic conditions (Supplementary Fig. 5B). The results indicated that accumulation of acetoin and glycerol might be determined by the degree of

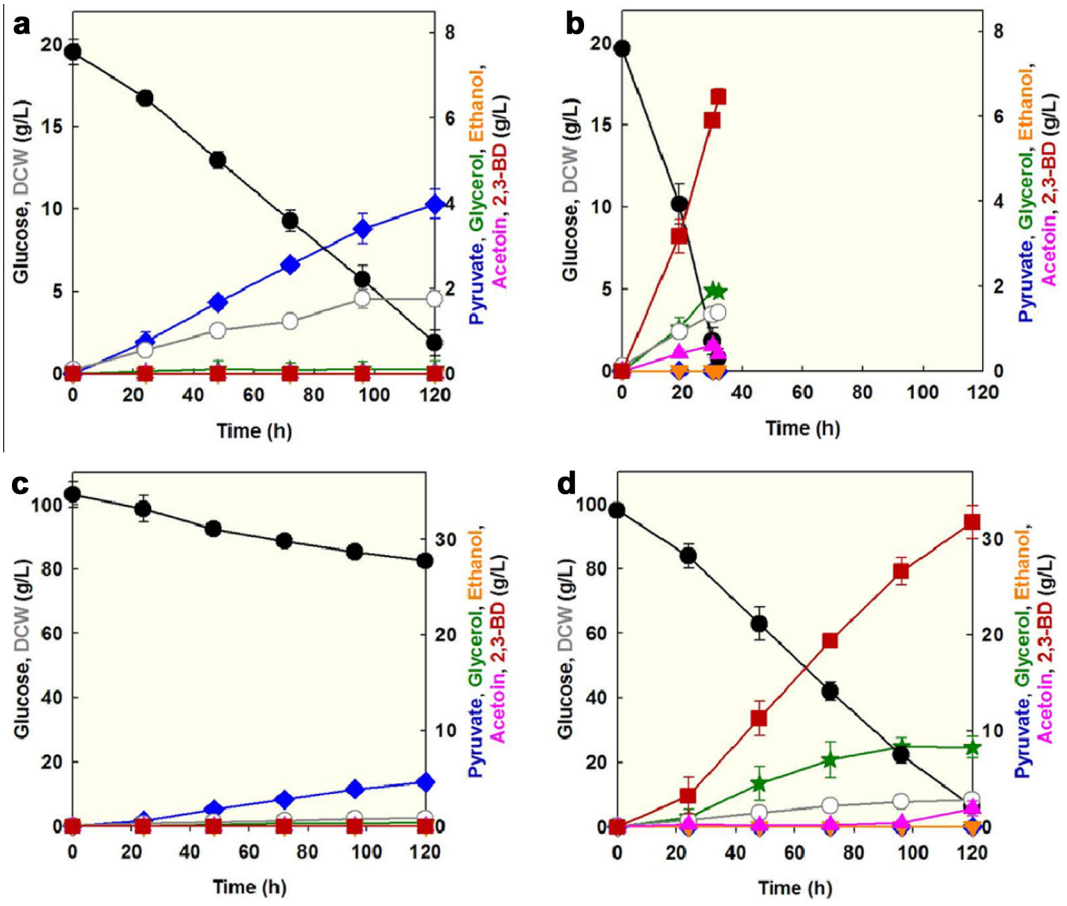

Fig. 3. Comparisons of glucose consumption and 2,3-BD production by BD4 (SOS4 with 2,3-BD producing plasmids) and CON (SOS4 with mother vector) under oxygen-limited condition. (a) CON in YP medium with 20 g/L of glucose; (b) BD4 in YP medium with 20 g/L of glucose; (c) CON in YP medium with 100 g/L of glucose; (d) BD4 in YP medium with 100 g/L of glucose. Symbols: glucose (circle), DCW (open circle), pyruvate (diamond), glycerol (star), ethanol (triangle down), acetoin (triangle up), and 2,3-BD (square). Error bars represent standard deviations associated with two independent experiments for 20 g/L of glucose and with three independent experiments for 100 g/L of glucose.

respiration, which can be controlled by aeration directly. As glucose represses respiration in *S. cerevisiae*, the amounts of supplied glucose is likely to affect the product formation pattern indirectly.

3.5. Effects of oxygen supply on 2,3-BD production in bioreactor

2,3-BD production from glucose is not redox neutral as two moles of NADH are produced in glycolysis but only one mole of NADH is oxidized to NAD^+ when one mole of glucose is converted into one mole of 2,3-BD. Therefore, oxygen supply for regenerating NAD^+ is the most important fermentation parameter for efficient production of 2,3-BD. Especially, alleviation of glycerol formation was observed in aerobic conditions, which suggested that increased respiration by oxygen might contribute to reoxidation of cytosolic NADH instead of formation of glycerol.

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 oxygen supply and 2,3-BD production, batch cultivations with 100 g/L of glucose was performed using a bioreactor under various aeration conditions (0, 0.25, 0.5, 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. 4). Glycerol accumulation reduced but ratios of acetoin increased with higher oxygen supply. 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. Among the aeration conditions tested, 1 vvm of aeration and 300 rpm of agitation were optimal for producing 2,3-BD by the BD4 strain using 100 g/L of glucose. The BD4 strain produced 34.8 g/L of 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 (Supplementary Fig. 6).

3.6. 2,3-BD production by BD4 strain in fed-batch fermentation

To determine the potential of the BD4 strain as a 2,3-BD producer, a fed-batch fermentation was carried out through intermittent addition of glucose (Fig. 5). 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).

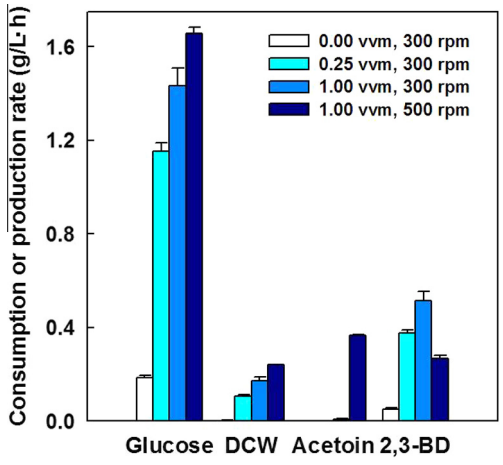

Fig. 4. Effect of aerations on product formation by the BD4 strain. Symbols: 0 vvm (white), 0.25 vvm (sky blue), 1.0 vvm (blue) with 300 rpm, and 1.0 vvm with 500 rpm (dark blue). Error bars represent standard deviations associated with two independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

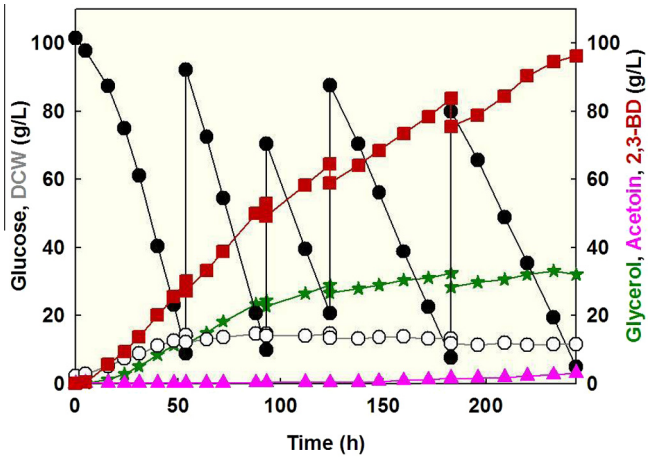

Fig. 5. 2,3-BD production during fed-batch fermentation of the BD4 strain. Symbols: glucose (circle), DCW (open circle), glycerol (star), acetoin (triangle up), and 2,3-BD (square).

Although promising results were obtained in this study, it is necessary to improve 2,3-BD productivity as high as those by native 2,3-BD producing bacteria through increasing glucose consumption and cell growth rates. Moreover, in order to improve the economics of the fermentation process, it is desirable to increase 2,3-BD yield through minimizing by-products formation.

4. Conclusions

An engineered *S. cerevisiae* strain (BD4) capable of producing 2,3-BD efficiently was constructed through elimination of pyruvate decarboxylase (*ΔPDC1* and *ΔPDC5*), introduction of acetolactate synthase and acetolactate decarboxylase (overexpression of *alsS* and *alsD*), and amplification of 2,3-butanediol dehydrogenase (overexpression of *BDH1*). 2,3-BD production by the BD4 strain was highly affected by aeration, indicating that redox maintenance is a key factor to produce 2,3-BD with high yields. The BD4 strain produced 96.2 g/L of 2,3-BD in a fed-batch fermentation under optimum conditions. These results suggest that engineered *S. cerevisiae* can be used for industrial production of 2,3-BD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.07.081.

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