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Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing

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ABSTRACT

2,3-Butanediol is a promising valuable chemical that can be used in various areas as a liquid fuel and a platform chemical. Here, 2,3-butanediol production in *Saccharomyces cerevisiae* was improved stepwise by eliminating byproduct formation and redox rebalancing. By introducing heterologous 2,3-butanediol biosynthetic pathway and deleting competing pathways producing ethanol and glycerol, metabolic flux was successfully redirected to 2,3-butanediol. In addition, the resulting redox cofactor imbalance was restored by overexpressing water-forming NADH oxidase (NoxE) from *Lactococcus lactis*. In a flask fed-batch fermentation with optimized conditions, the engineered *adh1Δadh2Δadh3Δadh4Δadh5Δgpd1Δgpd2Δ* strain overexpressing *Bacillus subtilis* α-acetolactate synthase (AlsS) and α-acetolactate decarboxylase (AlsD), *S. cerevisiae* 2,3-butanediol dehydrogenase (Bdh1), and *L. lactis* NoxE from a single multigene-expression vector produced 72.9 g/L 2,3-butanediol with the highest yield (0.41 g/g glucose) and productivity (1.43 g/(L · h)) ever reported in *S. cerevisiae*.

1. Introduction

Recently, microbial production of 2,3-butanediol has attracted great attention because of its extensive industrial applications as a platform chemical for the production of various derivatives such as 1,3-butadiene, methyl ethyl ketone (MEK), and diacetyl (Ji et al., 2011). Many bacterial species, including *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter aerogenes* and *Enterobacter cloacae*, can produce 2,3-butanediol efficiently with high titer and productivity (Celinska and Grajek, 2009; Jantama et al., 2015; Li et al., 2015). In these bacteria, two molecules of pyruvate are condensed to α-acetolactate by α-acetolactate synthase, and then α-acetolactate is further converted to acetoin through two different routes; direct conversion to acetoin by α-acetolactate decarboxylase or spontaneous decarboxylation to diacetyl, followed by conversion of diacetyl to acetoin by diacetyl reductase. Finally 2,3-butanediol dehydrogenase catalyzes the reduction of acetoin to 2,3-butanediol. However, because of the potential pathogenicity of these native producers, 2,3-butanediol production using these bacteria has been considered unsuitable for industrial-scale fermentation (Celinska and Grajek, 2009; Ji et al., 2011).

In this context, several efforts have been made to produce 2,3-butanediol in *S. cerevisiae* which is generally recognized as safe (GRAS) and widely used for the production of various chemicals and fuels because of its high tolerance to alcohols and harsh industrial conditions (Ansell et al., 1997; de Smidt et al., 2012; Hong and Nielsen, 2012; Hou et al., 2014). *S. cerevisiae* does not have α-acetolactate decarboxylase responsible for direct production of acetoin form α-acetolactate. Therefore, α-acetolactate, produced by α-acetolactate synthase (Ilv2) in mitochondria, is converted to acetoin via diacetyl formation (Supplementary Fig. S1). In addition, it has been reported that acetoin can be formed by the anomalous carboligase activity of pyruvate decarboxylase (PDC), which catalyzes condensation between pyruvate and acetaldehyde or two molecules of acetaldehyde (Chen and Jordan, 1984; Romano and Suzzi, 1996; Sergienko and Jordan, 2001). Then, acetoin is converted to 2,3-butanediol by the action of 2,3-butanediol dehydrogenase, Bdh1.

Because *S. cerevisiae* has a strong tendency toward ethanol fermentation, it is essential to reduce ethanol production and redirect this metabolic flux to 2,3-butanediol for high-yield production. Ethanol is produced from pyruvate via two steps, which consist of decarboxylation of pyruvate to acetaldehyde by PDC, and reduction of acetaldehyde to ethanol by alcohol dehydrogenase (ADH) (Fig. 1). Therefore, both PDC and ADH have been attractive disruption targets for 2,3-butanediol production in *S. cerevisiae*. In a previous study, deletion of *ADH1*, *ADH3*, and *ADH5* genes resulted in increased 2,3-butanediol production (2.29 g/L) with a yield of 0.113 g/g glucose under anaerobic condition (Ng et al., 2012). Although ethanol production can be completely eliminated by deleting *PDC1* and *PDC5* genes or all PDC genes (*PDC1*, *PDC5*, and *PDC6*), the resulting PDC-deficient strains have severe growth defects on glucose as a sole carbon source and

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require C_2 compounds such as acetate or ethanol for growth (Flikweert et al., 1996; Pronk et al., 1996). Therefore, adaptively evolved PDC-deficient strains overcoming these defects have been used for metabolic engineering applications (Kim et al., 2013; Lian et al., 2014; van Maris et al., 2004; Yan et al., 2014). Introduction of heterologous pathway consisting of α-acetolactate synthase (AlsS) and α-acetolactate decarboxylase (AlsD) from *B. subtilis* and endogenous Bdh1 to an evolved PDC-deficient strain led to successful production of 2,3-butanediol (96.2 g/L) with a high yield and productivity (0.28 g/g glucose and 0.39 g/(L · h), respectively) in fed-batch fermentation (Kim et al., 2013). Yeast strains producing 2,3-butanediol from xylose, galactose, or cellobiose apart from glucose, have also been developed (Kim et al., 2014; Lian et al., 2014; Nan et al., 2014).

It is also important to reduce or eliminate glycerol accumulation in terms of the efficient utilization of carbon source, because glycerol is one of the major products in *S. cerevisiae*. Especially, it has been reported that glycerol is further accumulated by disrupting ADH activity due to the increased demand for NAD^+ regeneration (Ida et al., 2012). Glycerol is produced from dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase (Fig. 1). *S. cerevisiae* has two isoforms of NAD^+-dependent glycerol-3-phosphate dehydrogenase, Gpd1 and Gpd2, which catalyze the reduction of DHAP to glycerol-3-phosphate (G3P) (Albertyn et al., 1994; Eriksson et al., 1995) (Fig. 1). In spite of the same catalytic function with similar kinetics, Gpd1 and Gpd2 are known to serve distinct physiological roles attributed by different transcriptional regulation (Ansell et al., 1997). Gpd1 is responsible for osmotic stress-induced glycerol production, whereas Gpd2 is involved in redox regulation. A number of studies have attempted to reduce glycerol formation for the production of ethanol, succinic acid, and lactic acid by deleting *GPD1* or/and *GPD2*, replacing the promoter sequences of *GPD1* and *GPD2* to the lower strength promoters, or deleting *FPS1*, which encodes a plasma membrane transporter of glycerol (Ding et al., 2013; Ida et al., 2013; Kong et al., 2006; Yan et al., 2014).

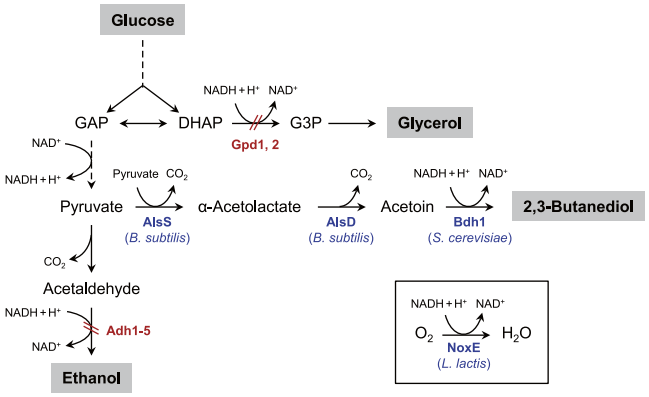

Fig. 1. Metabolic pathway for 2,3-butanediol production used in this study. Two molecules of pyruvate are converted to one molecule of 2,3-butanediol via α-acetolactate and acetoin by sequential actions of α-acetolactate synthase (AlsS), α-acetolactate decarboxylase (AlsD), and 2,3-butanediol dehydrogenase (Bdh1). The water-forming NADH oxidase (NoxE) catalyzes the oxidation of NADH to NAD^+ with the concomitant reduction of oxygen to water. Dashed arrows indicate multiple enzymatic steps. DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; GAP, glyceraldehyde-3-phosphate.

Redox cofactors, especially NAD^+/NADH pair, play an essential role in cellular metabolism by participating in a large number of biochemical reactions (Chen et al., 2014; Forster et al., 2003). Consequently, changes in cellular redox status, such as the ratio between NAD^+ and NADH, lead to widespread effects on metabolic network. Therefore, maintaining redox balance is not only a fundamental requirement for sustained cellular metabolism and cell growth, but also an important strategy for metabolic engineering (Heux et al., 2006). Metabolic cofactor imbalance caused by pathway engineering can be restored through removal of excess cofactor by enzymatic conversion using NADH oxidase or NADH kinase, or by modulating the cofactor specificity of pathway enzymes (Bastian et al., 2011; Hou et al., 2009; Zhang et al., 2014).

In this study, as an effort to develop *S. cerevisiae* strain for 2,3-butanediol production, we introduced 2,3-butanediol biosynthetic pathway into *S. cerevisiae* using a multigene-expression plasmid. To minimize byproduct formation, including ethanol and glycerol, genes encoding five alcohol dehydrogenases (*ADH1* to *ADH5*) and two glycerol-3-phosphate dehydrogenases (*GPD1* and *GPD2*) were deleted. Moreover, to relieve cofactor imbalance, water-forming NADH oxidase (NoxE) from *Lactococcus lactis* was expressed with 2,3-butanediol biosynthetic enzymes in *adh1-5Δgpd1Δgpd2Δ* strain, resulting in 2,3-butanediol production with dramatically improved productivity and high yield.

2. Materials and methods

2.1. Strains and media

All strains used in this study are listed in Table 1. *S. cerevisiae* CEN.PK2-1C was used as a parental strain. The gene disruption mutants were constructed by using the Cre/*loxP* recombination system (Gueldener et al., 2002). The gene deletion cassette was obtained by PCR amplification from pUG27 or pUG72 as template, using a gene-specific primer pair of d_ORF F and d_ORF R. After confirmation of the

Table 1
Strains used in this study.

Strain	Description	Genotype	Reference
CEN.PK2-1C	WT	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*	EUROSCARF
JHY601	*adh1Δ*	CEN.PK2-1C *adh1Δ::loxP*	(Kim and Hahn, 2014)
JHY602	*adh1-5Δ*	CEN.PK2-1C *adh1Δ::loxP adh2Δ::loxP adh3Δ::loxP adh4Δ::loxP adh5Δ::loxP*	This study
JHY603	*gpd1Δgpd2Δ*	CEN.PK2-1C *gpd1Δ::loxP gpd2Δ::loxP*	This study
JHY604	*adh1Δgpd1Δgpd2Δ*	CEN.PK2-1C *adh1Δ::loxP gpd1Δ::loxP gpd2Δ::loxP*	This study
JHY605	*adh1-5Δgpd1Δgpd2Δ*	CEN.PK2-1C *adh1Δ::loxP adh2Δ::loxP adh3Δ::loxP adh4Δ::loxP adh5Δ::loxP gpd1Δ::loxP gpd2Δ::loxP*	This study
JHY606	WT [C]	CEN.PK2-1C harboring p413GPD	This study
JHY607	WT [SDB]	CEN.PK2-1C harboring p413-SDB	This study
JHY608	*adh1Δ* [SDB]	JHY601 harboring p413-SDB	This study
JHY609	*adh1-5Δ* [SDB]	JHY602 harboring p413-SDB	This study
JHY610	*gpd1Δgpd2Δ* [SDB]	JHY603 harboring p413-SDB	This study
JHY611	*adh1Δgpd1Δgpd2Δ* [SDB]	JHY604 harboring p413-SDB	This study
JHY612	*adh1-5Δgpd1Δgpd2Δ* [SDB]	JHY605 harboring p413-SDB	This study
JHY613	*adh1-5Δgpd1Δgpd2Δ* [SDBN]	JHY605 harboring p413-SDBN	This study

Table 2
Plasmids used in this study.

Plasmid	Description	Reference
pUG27	Plasmid containing *loxP-his5*$^+$*-loxP* deletion cassette	EUROSCARF
pUG72	Plasmid containing *loxP-URA3-loxP* deletion cassette	EUROSCARF
pSH63	*TRP1*, Cre recombinase under the control of *GAL1* promoter	EUROSCARF
p413GPD	CEN/ARS plasmid, *HIS3*, P_{TDH3}, T_{CYC1}	(Mumberg et al., 1995)
p413-SDB	CEN/ARS plasmid, *HIS3*, P_{TDH3}-*alsS*-T_{PYK1}, P_{TEF1}-*alsD*-T_{GPM1}, P_{TPI1}-*BDH1*-T_{TPI1}	This study
p413-SDBN	CEN/ARS plasmid, *HIS3*, P_{TDH3}-*alsS*-T_{PYK1}, P_{TEF1}-*alsD*-T_{GPM1}, P_{TPI1}-*BDH1*-T_{TPI1}, P_{FBA1}-*noxE*-T_{FBA1}	This study

correct integration of the cassette at the target gene locus through PCR analysis using the confirmation primers (c_ORF F and c_ORF R), the marker gene was removed by transformation of Cre recombinase-expression vector, pSH63. Additional gene deletion was sequentially conducted using the same procedure. Primers used for gene deletion are listed in Supplementary Table S1.

Yeast cells were cultured in YP medium (10 g/L yeast extract and 20 g/L bacto-peptone) supplemented with 20 (YPD), 50 (YPD5), or 100 g/L glucose (YPD10) or in synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids, 1.4 g/L amino acids dropout mixture lacking His, Trp, Leu, and Ura) supplemented with auxotrophic amino acids as required and 20 or 50 g/L glucose.

2.2. Plasmid construction

Plasmids used in this study are listed in Table 2. For gene overexpression, strong constitutive promoters (P_{TPI1} and P_{FBA1}) and terminators (T_{PYK1}, T_{GPM1}, T_{TPI1}, and T_{FBA1}) were prepared by PCR amplification using CEN.PK2-1C genomic DNA as template. The *TDH3* promoter of p414GPD was removed by cutting the plasmid with SacI and SpeI, and replaced with the DNA fragments P_{TPI1} and P_{FBA1}, generating p414_P_{TPI1} and p414_P_{FBA1}. The *CYC1* terminators of p414GPD (containing P_{TDH3}), p414TEF (containing P_{TEF1}), p414_P_{TPI1}, and p414_P_{FBA1} were removed by cutting the plasmids with XhoI and KpnI, and replaced with the DNA fragments T_{PYK1}, T_{GPM1}, T_{TPI1}, and T_{FBA1}, respectively, generating p414_P_{TDH3}/T_{PYK1}, p414_P_{TEF1}/T_{GPM1}, p414_P_{TPI1}/T_{TPI1}, and p414_P_{FBA1}/T_{FBA1}.

The *alsS* and *alsD* genes from *B. subtilis*, *BDH1* from *S. cerevisiae*, and *noxE* from *L. lactis* IL1403 were amplified by PCR using each genomic DNA. These PCR products were cloned into appropriate plasmids, resulting in p414_P_{TDH3}-*alsS*-T_{PYK1}, p414_P_{TEF1}-*alsD*-T_{GPM1}, p414_P_{TPI1}-*BDH1*-T_{TPI1}, and p414_P_{FBA1}-*noxE*-T_{FBA1}. To construct multigene-expression vector for 2,3-butanediol pathway, the *alsD*-expression cassette (P_{TEF1}-*alsD*-T_{GPM1}) flanked by MluI sites was obtained by PCR amplification using a universal primer pair, Univ F and Univ R containing AscI and NotI sites for additional cloning, and cloned into the BssHII sites of pRS413 vector. *BDH1*- and *alsS*-expression cassettes flanked by MluI and NotI sites were also obtained using the same primers and sequentially cloned into AscI and NotI sites, resulting in p413-SDB (Fig. 2). The *noxE*-expression cassette (P_{FBA1}-*noxE*-T_{FBA1}) was additionally cloned into AscI and NotI sites of p413-SDB, resulting in p413-SDBN (Fig. 2). Primers used for gene cloning are listed in Supplementary Table S2.

2.3. Fermentation experiments

Yeast cells harboring appropriate plasmids were pre-cultured in SC-His medium containing 20 g/L glucose and diluted to OD_{600} of 0.3 in 8 mL of SC-His medium containing 50 g/L glucose in a 50 mL conical tube, and then cultivated at 30 °C with shaking at 170 rpm. For shake flask fed-batch fermentation, cells harboring p413-SDBN plasmid were pre-cultured in SC-His medium containing 20 g/L glucose, diluted to OD_{600} of 10, and cultured in 25 mL of YPD10 medium in a 250 mL flask at 30 °C with shaking at 170 rpm. Glucose was intermittently added to the culture medium by using the feeding solution, which was prepared by dissolving 800 g of glucose in 1 L of water.

2.4. Analytical methods

To quantify the concentration of metabolites, 800 μL of culture supernatants were collected and filtered through a 0.22 μm syringe filter. High performance liquid chromatography (HPLC) analysis was performed in UltiMate 3000 HPLC system (Thermo fishers scientific) equipped with a BioRad Aminex HPX-87H column (300 mm × 7.8 mm, 5 μm) at 60 °C with 5 mM H_2SO_4 as a flow rate of 0.6 mL/min and refractive index (RI) detector.

3. Results and discussion

3.1. Construction of 2,3-butanediol biosynthetic pathway in *S. cerevisiae*

S. cerevisiae has an innate 2,3-butanediol production pathway, but the efficiency is very low. To enhance 2,3-butanediol production, heterologous acetoin biosynthetic pathway in *B. subtilis*, consisting of α-acetolactate synthase (AlsS) and α-acetolactate decarboxylase (AlsD), was introduced into *S. cerevisiae* which lacks α-acetolactate decarboxylase. Pyruvate is sequentially converted to α-acetolactate and acetoin by AlsS and AlsD, respectively, and then acetoin can be converted to 2,3-butanediol by endogenous 2,3-butanediol dehydrogenase (Bdh1) (Fig. 1). This pathway has been successfully adopted to produce 2,3-butanediol in *S. cerevisiae* and *Escherichia coli* in previous studies (Kim and Hahn, 2014; Kim et al., 2013; Nakashima et al., 2014).

To construct multigene-expression plasmid for the expression of genes required for 2,3-butanediol biosynthesis, we developed multiple cloning system based on isocaudomer restriction enzyme pairs (BssHII-MluI and AscI-MluI) using a CEN/ARS-based low copy number plasmid (pRS413) as a vector backbone (Fig. 2). The resulting plasmid p413-SDB is composed of *alsS*, *alsD*, and *BDH1* genes under the control of strong constitutive promoters, P_{TDH3}, P_{TEF1}, and P_{TPI1}, respectively, and different terminators.

We verified the effect of introducing 2,3-butanediol pathway into *S. cerevisiae* by transforming p413-SDB or empty p413GPD plasmid into CEN.PK2-1C strain. The control strain harboring p413GPD (WT [C]) produced only a trace amount of 2,3-butanediol (< 0.1 g/L), mainly producing ethanol with a titer of 22.0 g/L after 24 h fermentation in SC-His medium containing 50 g/L glucose (Fig. 3A). Whereas, CEN.PK2-1C strain harboring p413-SDB plasmid (WT [SDB]) produced up to 11.3 g/L 2,3-butanediol as a major product, successfully competing with the pyruvate flux to ethanol (Fig. 3B). As a result, ethanol production was significantly reduced to 6.0 g/L, but glycerol was accumulated up to 6.6 g/L (Fig. 3B). In 2,3-butanediol production pathway, two molecules of NAD^+ are consumed in glycolysis to generate two molecules of pyruvate, but only one molecule of NAD^+ is regenerated from two molecules of pyruvate by reducing acetoin to 2,3-butanediol (Fig. 1).

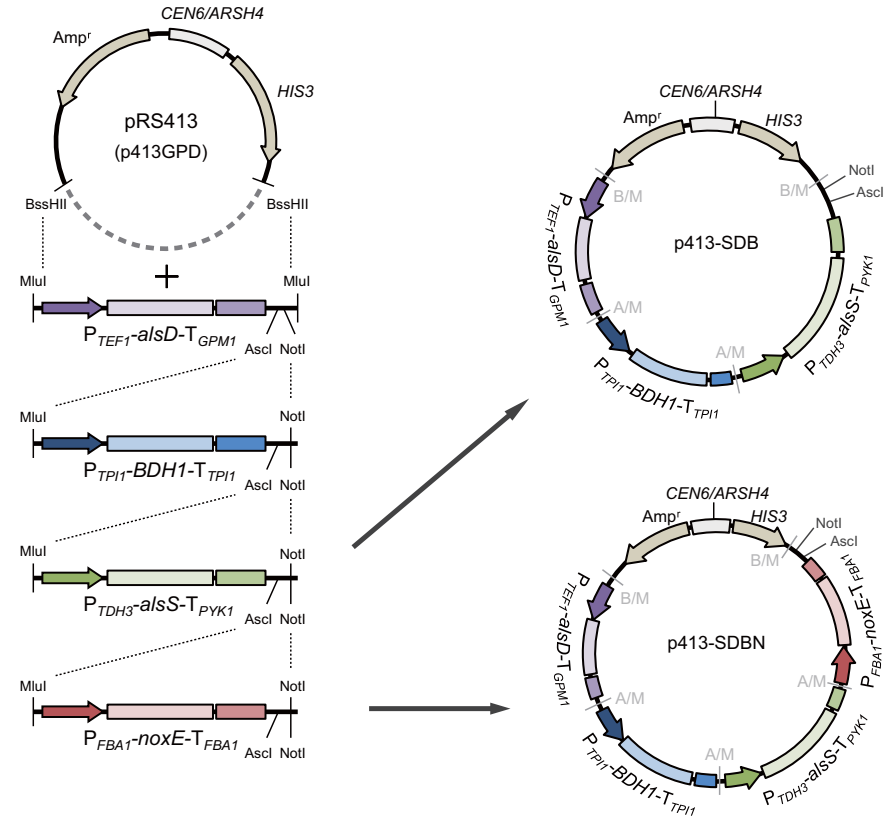

Fig. 2. Construction of multigene-expression vector. Gene-expression cassettes flanked by MluI and AscI-NotI-MluI sites were obtained by PCR using primers carrying the restriction enzyme sites. First, MluI-digested cassette was cloned into the BssHII sites of pRS413 vector, resulting in uncleavable BssHII-MluI ligation site (B/M) and AscI/NotI site for additional cloning. Additional cloning were sequentially carried out by ligating AscI/NotI-digested vector and MluI/NotI-digested gene expression cassette, resulting in uncleavable AscI-MluI ligation site (A/M), and new cloning sites (AscI and NotI sites). The experimental details are described in Section 2.

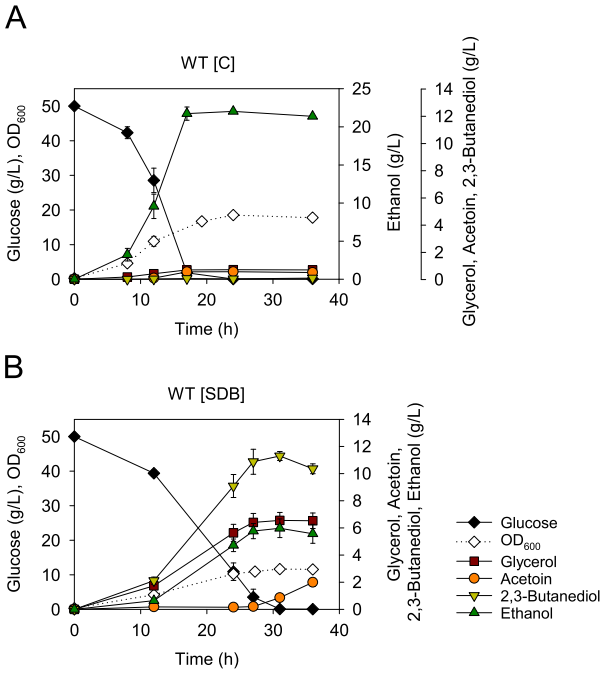

Fig. 3. Improvement of 2,3-butanediol production by introducing 2,3-butanediol biosynthetic pathway in *S. cerevisiae*. The yeast strains WT [C] (A) and WT [SDB] (B) were grown in SC-His media containing 50 g/L glucose. Error bars indicate standard deviations of four independent experiments.

Therefore, introduction of metabolic pathway to produce 2,3-butanediol from glucose leads to redox cofactor imbalance, resulting in glycerol accumulation as a compensation mechanism to regenerate NAD^+ (Bakker et al., 2001) (Fig. 3). Taken together, the pyruvate flux was dramatically reconstructed to produce 2,3-butanediol just by overexpressing *alsS* and *alsD* genes from *B. subtilis* and native *BDH1* gene using multigene-expression plasmid.

2,3-Butanediol production pathway was reversed after glucose depletion, exhibiting a gradual conversion of 2,3-butanediol to acetoin (Fig. 3B). The factors deriving the forward reaction, the continuous supply of acetoin and demand for NAD^+ regeneration for glycolysis, might be inactivated upon the termination of glycolysis, resulting in the reversion of the reaction toward acetoin formation by using abundant substrate, 2,3-butanediol. However, the total concentrations of 2,3-butanediol and acetoin remained unchanged, suggesting that acetoin is not further metabolized for the respiratory growth.

3.2. Disruption of competing pathways to improve 2,3-butanediol production

In WT [SDB] strain, ethanol and glycerol are the major byproducts. Therefore, to further improve 2,3-butanediol production, it is critical to minimize the metabolic pathways producing these byproducts. Although deletion of PDC could be the most efficient way of eliminating the competing pyruvate flux to ethanol, utilization of PDC-deficient strains is limited because of their severe growth defects in glucose medium. The growth defects of PDC-deficient strains are in part due to the limitation of producing cytosolic acetyl-CoA from acetaldehyde via acetate (Flikweert et al., 1996). The problem of cytosolic acetyl-CoA can be solved by eliminating ADH instead of PDC, but the accumulation of toxic aldehyde could be a potential problem in ADH-deficient strains. However, the efficient redirection of pyruvate flux to 2,3-butanediol in WT [SDB] strain (Fig. 3B) suggests that AlsS activity might be high enough to compete with PDC, thus

minimizing the accumulation of acetaldehyde even in the absence of ADH. Therefore, we chose to delete ADH genes instead of PDC genes to reduce ethanol production. In *S. cerevisiae*, there are at least five isozymes of NAD^+-dependent alcohol dehydrogenase, Adh1 to Adh5, among which Adh1 is known as the major enzyme (de Smidt et al., 2008; Leskovac et al., 2002). To reduce ethanol production, we first constructed *ADH1* deletion strain and investigated the effect on metabolites profile. The *adh1Δ* strain showed slow growth rate (Supplementary Fig. S2A) because of the accumulation of NADH and acetaldehyde as previously reported (de Smidt et al., 2012; Drewke et al., 1990; Ng et al., 2012). When 2,3-butanediol pathway was introduced into *adh1Δ* strain (*adh1Δ* [SDB]), the growth defect was considerably recovered (Fig. 4A), which may be due to NAD^+-regeneration by Bdh1 and pyruvate flux to α-acetolactate instead of acetaldehyde formation. The *adh1Δ* [SDB] strain produced 3.4 g/L ethanol after 27 h (Fig. 4A), indicating a 43% decrease compared with WT [SDB] strain (Fig. 3B). On the other hand, both 2,3-butanediol and glycerol production increased by 18% and 30%, reaching 13.3 g/L and 8.6 g/L, respectively, compared with WT [SDB] strain. The increase in glycerol production can be explained as a compensation mechanism of regenerating NAD^+ to maintain redox balance (Bakker et al., 2001). When all five ADH genes were deleted (*adh1-5Δ*), ethanol production was almost completely blocked (<0.4 g/L) (Fig. 4B). However, *adh1-5Δ* [SDB] strain showed similar level of 2,3-butanediol production compared with *adh1Δ* [SDB] strain, while exhibiting further increase in glycerol production by 22% (10.5 g/L after 36 h).

In addition, we investigated the effect of deleting glycerol pathway on 2,3-butanediol production. Since glycerol-3-phosphate dehydrogenase is the rate-controlling enzyme in the glycerol production (Cronwright et al., 2002), we deleted both *GPD1* and *GPD2* to block the carbon flux to glycerol. In agreement with previous reports (Ansell et al., 1997; Guadalupe Medina et al., 2010), glycerol accumulation was completely eliminated in *gpd1Δgpd2Δ* strain containing p413-SDB plasmid (*gpd1Δgpd2Δ* [SDB]) (Fig. 4C). In

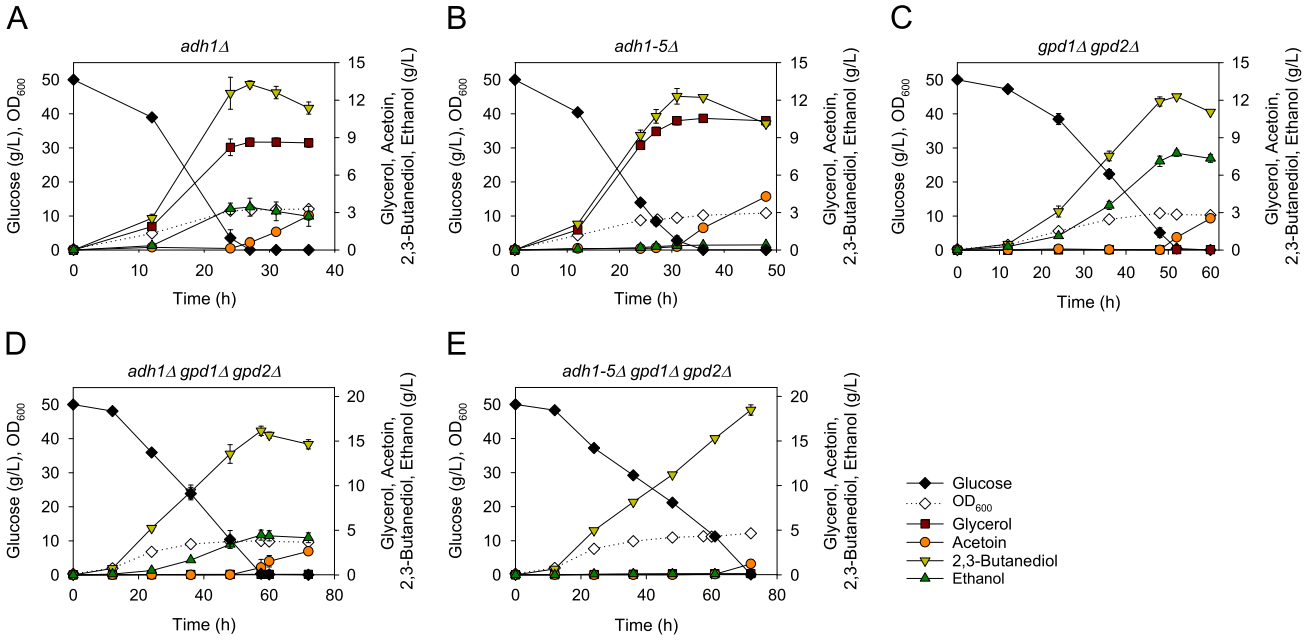

Fig. 4. The effect of deleting competing pathways on 2,3-butanediol production. The yeast strains *adh1Δ* [SDB] (A), *adh1-5Δ* [SDB] (B), *gpd1Δgpd2Δ* [SDB] (C), *adh1Δgpd1Δgpd2Δ* [SDB] (D), and *adh1-5Δgpd1Δgpd2Δ* [SDB] (E) were grown in SC-His containing 50 g/L glucose. Error bars indicate standard deviations of four independent experiments.

Table 3
Fermentation characteristics of recombinant strains.

Strain	Description	Fermentation time (h)	Products (g/L)					Productivity of 2,3-BDO (g/(L·h))	Yield of 2,3-BDO (g/g glucose)
			Ethanol	Glycerol	Acetoin	2,3-BDO	Acetoin +2,3-BDO		
Batch flask fermentation									
JHY606	WT [C]	24	22.03 ± 0.11	0.70 ± 0.01	0.56 ± 0.05	0.03 ± 0.00	0.59 ± 0.05	0.001 ± 0.000	0.001 ± 0.000
JHY607	WT [SDB]	31	5.97 ± 0.69	6.55 ± 0.58	0.86 ± 0.24	11.30 ± 0.33	12.16 ± 0.43	0.365 ± 0.011	0.226 ± 0.007
JHY608	*adh1Δ* [SDB]	27	3.44 ± 0.72	8.64 ± 0.32	0.60 ± 0.37	13.27 ± 0.28	13.87 ± 0.59	0.492 ± 0.010	0.266 ± 0.006
JHY609	*adh1-5Δ* [SDB]	36	0.39 ± 0.05	10.54 ± 0.25	1.77 ± 0.15	12.22 ± 0.13	14.00 ± 0.25	0.340 ± 0.004	0.245 ± 0.003
JHY610	*gpd1Δgpd2Δ* [SDB]	52	7.76 ± 0.25	0.04 ± 0.00	1.02 ± 0.19	12.29 ± 0.12	13.31 ± 0.24	0.236 ± 0.002	0.248 ± 0.003
JHY611	*adh1Δgpd1Δgpd2Δ* [SDB]	60	4.38 ± 0.57	0.07 ± 0.01	1.54 ± 0.65	15.67 ± 0.34	17.20 ± 0.34	0.261 ± 0.006	0.314 ± 0.007
JHY612	*adh1-5Δgpd1Δgpd2Δ* [SDB]	72	0.07 ± 0.02	0.13 ± 0.00	1.22 ± 0.45	18.47 ± 0.58	19.69 ± 0.14	0.257 ± 0.008	0.370 ± 0.011
JHY613	*adh1-5Δgpd1Δgpd2Δ* [SDBN]	43	0.12 ± 0.09	0.04 ± 0.01	1.78 ± 0.22	18.89 ± 1.22	20.67 ± 1.00	0.442 ± 0.029	0.380 ± 0.023
Fed-batch flask fermentation									
JHY613	*adh1-5Δgpd1Δgpd2Δ* [SDBN]	51	0.31 ± 0.16	0.34 ± 0.05	1.38 ± 0.56	72.91 ± 4.70	74.29 ± 4.31	1.430 ± 0.092	0.407 ± 0.010

comparison with WT [SDB] (Fig. 3B), 2,3-butanediol titer increased from 11.3 to 12.3 g/L, but ethanol titer also increased from 6.0 g/L to 7.8 g/L.

Next, we investigated 2,3-butanediol production upon deletion of both ethanol and glycerol pathways. In *adh1Δ* and *adh1-5Δ* strains, both *GDP1* and *GPD2* genes were additionally deleted to generate *adh1Δgpd1Δgpd2Δ* and *adh1-5Δgpd1Δgpd2* strains. Additional deletion of ADH genes in *gpd1Δgpd2Δ* strain led to a decrease in ethanol production, and the reduced ethanol levels in *adh1Δgpd1Δgpd2Δ* [SDB] and *adh1-5Δgpd1Δgpd2* [SDB] strains contributed to the increase in 2,3-butanediol production levels accordingly (Fig. 4D and E). The final *adh1-5Δgpd1Δgpd2* [SDB] strain produced up to 18.5 g/L 2,3-butanediol with a yield of 0.37 g/g glucose after 72 h, reaching 74% of maximum theoretical yield (Fig. 4E and Table 3). In addition to high 2,3-butanediol yield, byproduct formation was dramatically reduced in this strain (0.07 g/L ethanol and 0.13 g/L glycerol).

3.3. Recovering redox imbalance by expressing water-forming NADH oxidase noxE

The cofactor imbalance generated during the 2,3-butanediol production from glucose can be relieved by NAD^+ regeneration through glycerol production (Fig. 3). However, as we eliminated the glycerol pathway to increase 2,3-butanediol production yield, glucose consumption rate and 2,3-butanediol productivity were considerably reduced in the *gpd1Δgpd2* background strains, coinciding with increases in 2,3-butanediol titer and yield (Fig. 4 and Table 3).

To alleviate the redox imbalance and improve the fermentation performance, the *noxE* gene from *L. lactis* encoding the water-forming NADH oxidase, which provides extra route for NAD^+ regeneration, was coupled to 2,3-butanediol biosynthetic pathway (Fig. 1). The water-forming NADH oxidase regenerates NAD^+ from NADH by using molecular oxygen. Previous studies have demonstrated that NoxE mainly localizes in the cytosol in *S. cerevisiae* and has high affinity for NADH, which provides competitive advantage against endogenous NADH-dependent enzymes (Heux et al., 2006; Vemuri et al., 2007). Introducing *noxE* gene into xylose-utilizing *S. cerevisiae* led to increased ethanol production and decreased glycerol and xylitol accumulation (Hou et al., 2014; Zhang et al., 2012). In addition, cofactor engineering using NADH oxidase has been successfully applied to acetoin production in *B. subtilis*, *K. pneumonia*, and *Serratia marcescens* (Ji et al., 2013; Sun et al., 2012; Zhang et al., 2014).

The *noxE* gene under the control of strong constitutive promoter, P_{FBA1}, was cloned into p413-SDB plasmid, generating p413-SDBN plasmid (Fig. 2). The *adh1-5Δgpd1Δgpd2* strain harboring p413-SDBN plasmid (*adh1-5Δgpd1Δgpd2* [SDBN]) showed significant improvement in 2,3-butanediol productivity (0.44 g/(L·h)) compared with *adh1-5Δgpd1Δgpd2* [SDB] strain (0.26 g/(L·h)), while exhibiting similar levels of both 2,3-butanediol titer and yield (Fig. 5 and Table 3). This result indicates that redox imbalance caused by 2,3-butanediol production can be successfully restored by expressing NADH oxidase. During the review process of this manuscript, the effect of NADH oxidase on 2,3-butanediol production have also been reported in the PDC-deficient *S. cerevisiae* strain (Kim et al., 2015).

3.4. Fed-batch fermentation for 2,3-butanediol production

In order to further improve the fermentation performance to produce 2,3-butanediol in *adh1-5Δgpd1Δgpd2* [SDBN] strain (JHY613), various culture conditions were investigated. 2,3-Butanediol productivity was improved by increasing the initial cell density from OD_{600} of 0.3 to 10 (Fig. 5 and Supplementary Fig. S3A). Similar to a previous report (Lian et al., 2014),

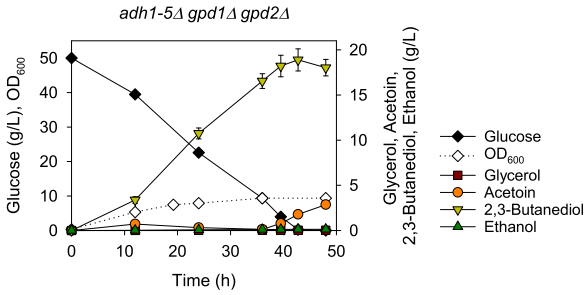

Fig. 5. The effect of *noxE* expression on 2,3-butanediol production. The *adh1-5Δgpd1Δgpd2Δ* [SDBN] was grown in SC-His containing 50 g/L glucose. Error bars indicate standard deviations of four independent experiments.

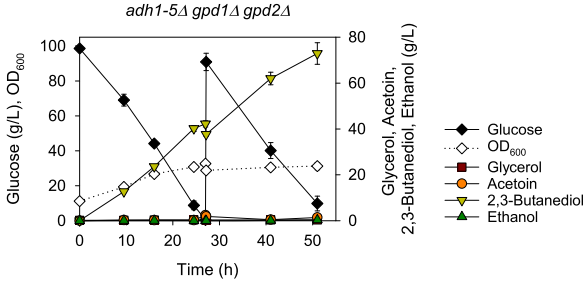

Fig. 6. 2,3-Butanediol fermentation profile in shake flask with glucose feeding. *adh1-5Δ gpd1Δgpd2Δ* [SDBN] was grown in YPD10 containing 100 g/L glucose with initial OD_{600} of 10. The feeding solution (800 g/L glucose) was added to the culture medium when glucose was depleted (after 27 h fermentation). Error bars indicate standard deviations of four independent experiments.

fermentation in YPD medium exhibited slightly higher 2,3-butanediol productivity than that of in SC medium (Supplementary Fig. S3). When aeration was increased by growing cells with 25 ml culture volume in a 250 ml flask, 2,3-butanediol productivity was remarkably improved compared with that obtained from the routine cultivation condition of 8 ml culture in a 50 ml conical tube (Supplementary Fig. S4), suggesting that increased aeration might contribute to NAD^+ regeneration through respiration or increasing NoxE activity. Although NoxE has very low K_m constant for oxygen (Sudar et al., 2014), oxygen concentration might affect the NoxE activity in the fermentation. Taken together, flask fed-batch fermentation was carried out in YPD10 medium containing 100 g/L glucose with initial high cell density of OD_{600} of 10. After 51 h fermentation, 72.9 g/L 2,3-butanediol was produced with a yield of 0.41 g/g glucose, reaching 81.4% of maximum theoretical yield (Fig. 6). Furthermore, 2,3-butanediol productivity was dramatically improved to 1.43 g/(L·h). Although 2,3-butanediol titer (72.9 g/L) was lower than that of previous reports (above 100 g/L) (Kim et al., 2013; Lian et al., 2014), JHY613 strain achieved the highest yield and productivity ever reported in *S. cerevisiae*.

4. Conclusions

In this study, we developed an engineered *S. cerevisiae* strain for efficient 2,3-butanediol production by deleting competing pathways producing ethanol and glycerol and introducing biosynthetic pathway consisting of AlsS and AlsD from *B. subtilis* and endogenous Bdh1, and NADH oxidase (NoxE) from *L. lactis* for redox rebalance. In previous reports to produce 2,3-butanediol in *S. cerevisiae* strains with reduced ethanol production, significant amount of glycerol was accumulated as a regulatory mechanism to maintain redox balance, leading to a decrease in 2,3-butanediol yield (Kim et al., 2014; Ng et al., 2012). Although some attempts have been made to minimize glycerol accumulation by optimizing

aeration conditions, glycerol production was considered as an inevitable phenomenon during 2,3-butanediol production in *S. cerevisiae* (Kim et al., 2013; Kim et al., 2014; Ng et al., 2012). However, in this study, glycerol accumulation was completely eliminated by deleting both *GPD1* and *GPD2* genes, and the resulting redox imbalance was successfully relieved by coupling the NAD^+-regenerating NADH oxidase to 2,3-butanediol production pathway. By using these strategies, we could achieve both high yield and productivity in 2,3-butanediol production. In our final strain JHY613, 2,3-butanediol was produced up to 72.9 g/L after 51 h cultivation, with 84% of the maximum theoretical yield. To further improve 2,3-butanediol production, future works should be focused on further optimization of fermentation conditions and improvement of cellular tolerance to 2,3-butanediol.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.07.006.

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