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Expression of *Lactococcus lactis* NADH oxidase increases 2,3-butanediol production in Pdc-deficient *Saccharomyces cerevisiae*

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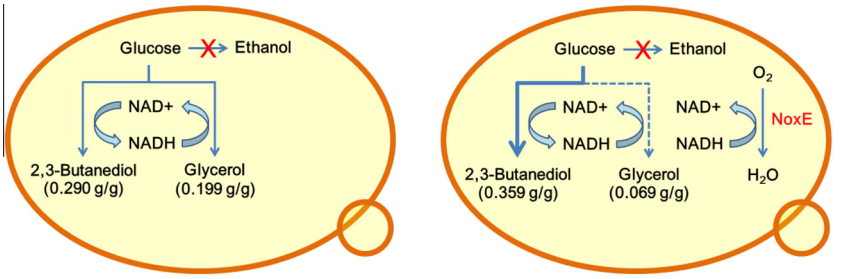
HIGHLIGHTS

- *Saccharomyces cerevisiae* was engineered to produce 2,3-butanediol (2,3-BD).
- The engineered Pdc-deficient *S. cerevisiae* could grow on synthetic glucose medium.
- The small amount of ethanol supplemented greatly improved 2,3-BD formation.
- Expression of NADH oxidase substantially increased the 2,3-BD yield.

GRAPHICAL ABSTRACT



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ABSTRACT

To minimize glycerol production during 2,3-BD fermentation by the engineered *Saccharomyces cerevisiae*, the *Lactococcus lactis* water-forming NADH oxidase gene (*noxE*) was expressed at five different levels. The expression of NADH oxidase substantially decreased the intracellular NADH/NAD^+ ratio. The *S. cerevisiae* BD5_T2nox strain expressing *noxE* produced 2,3-BD with yield of 0.359 g 2,3-BD/g glucose and glycerol with 0.069 g glycerol/g glucose, which are 23.8% higher and 65.3% lower than those of the isogenic strain without *noxE*. These results demonstrate that the carbon flux could be redirected from glycerol to 2,3-BD through alteration of the NADH/NAD^+ ratio by the expression of NADH oxidase.

1. Introduction

2,3-Butanediol (2,3-BD) is a versatile chemical that has various applications to chemical industries fields. 2,3-BD can be used as an anti-freeze agent and solvent (Syu, 2001). 2,3-BD can also be chemically-modified to produce other chemicals. Among the dehydrated compounds of 2,3-BD, methylethylketone (MEK) is considered to be an effective additive of liquid fuel (Tran and Chambers, 1987), and 1,3-butadiene can be used to make synthetic rubber (Syu, 2001). Polyurethane forms can be made by the reaction of 2,3-BD and boric acid (Paciorek-Sadowska and Czuprynski, 2006).

A number of studies on the production of 2,3-BD with bacterial strains such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Serratia marcescens* have been reported (Petrov and Petrova, 2009; Qureshi and Cheryan, 1989; Zhang et al., 2010). Alternatively, *Saccharomyces cerevisiae* could be a promising host strain for the production of 2,3-BD for industrial applications because *S. cerevisiae* is a generally recognized as safe (GRAS) microorganism and large scale fermentation technologies for *S. cerevisiae* have been well-developed. A wild-type *S. cerevisiae* produces trace

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amounts of 2,3-BD (0.4–2.0 g/L) during wine fermentation (Ehsani et al., 2009) although *S. cerevisiae* possesses three putative pathways converting pyruvate to 2,3-BD (Romano and Suzzi, 1996). Metabolic engineering to produce 2,3-BD with engineered *S. cerevisiae* strains harboring the bacterial 2,3-BD biosynthetic pathway have been attempted (Kim et al., 2013; Ng et al., 2012). The bacterial 2,3-BD biosynthetic pathway converts pyruvate into 2,3-BD by three-step enzyme reactions: two molecules of pyruvate are condensed to produce α-acetolactate by acetolactate synthase, α-acetolactate is decarboxylated to produce acetoin by acetolactate decarboxylase, and acetoin is further reduced into 2,3-BD by butanediol dehydrogenase. As pyruvate is also a precursor of ethanol in yeast, it is crucial to limit metabolic fluxes toward ethanol production for the efficient production of 2,3-BD. Either alcohol dehydrogenase or pyruvate decarboxylase reactions can be perturbed to eliminate ethanol production in yeast (Kim et al., 2013). The engineered *S. cerevisiae* BY4742 *Δadh1Δadh3Δadh5* expressing *Bacillus subtilis* acetolactate synthase (*alsS*), *Enterobacter aerogenes* acetolactate decarboxylase (*budA*), and *E. aerogenes* butanediol dehydrogenase (*budC*) produced 2.29 g/L of 2,3-BD with a yield of 0.113 g 2,3-BD/g glucose (Ng et al., 2012). Recently, high titer production (96.2 g/L) of 2,3-BD was achieved using a pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* strain expressing acetolactate synthase (*alsS*) and acetolactate decarboxylase (*alsD*) from *B. subtilis* and overexpressing the endogenous 2,3-butanediol dehydrogenase 1 (*BDH1*). However, substantial amounts of glycerol were produced in parallel with 2,3-BD formation due to redox imbalance by excess production of cytosolic NADH under oxygen-limited conditions (Kim et al., 2013). In addition to glucose, 2,3-BD production from cellulosic sugars, such as cellobiose and xylose by engineered yeast have been reported (Kim et al., 2014; Nan et al., 2014).

The excess production of cytosolic NADH is caused by rerouting metabolic fluxes from ethanol to 2,3-BD formation. In a wild-type *S. cerevisiae*, ethanol production from glucose is a redox neutral process because NADH produced by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be re-oxidized from the conversion of pyruvate into ethanol. In the 2,3-BD production, on the other hand, 2 moles of pyruvate produced by glycolysis were converted into 1 mole of 2,3-BD with only 1 mole of NADH oxidized (Syu, 2001). As a result, excess cytosolic NADH can accumulate in the 2,3-BD-producing Pdc-deficient *S. cerevisiae*. In *S. cerevisiae*, glycerol functions mainly as a redox sink by balancing the net NADH surplus under oxygen-limited conditions (Ansell et al., 1997). The cytosolic NADH could be reoxidized to NAD^+ by glycerol formation through the reduction of dihydroxyacetone phosphate by glycerol 3-phosphate dehydrogenase (*GPD*) and the dephosphorylation of glycerol 3-phosphate by glycerol 3-phosphate phosphatase (*GPP*) (Costenoble et al., 2000). Synthesis of 1 mole of glycerol can reoxidize 1 mole of NADH. Therefore, a significant amount of glycerol is generated by the 2,3-BD-producing Pdc-deficient *S. cerevisiae* to balance the cytosolic redox state in oxygen-limited conditions (Kim et al., 2013, 2014; Nan et al., 2014). Removal of excess cytosolic NADH by other metabolic reactions could change the carbon flux from glycerol to 2,3-BD in the 2,3-BD-producing Pdc-deficient *S. cerevisiae*.

There have been numerous attempts to alter cytosolic concentrations of NADH in *S. cerevisiae* through interconverting between NADH and NADPH, or by using the accumulation of metabolites capable of being reduced or oxidized. These include expressions of the ammonium assimilation enzymes (Nissen et al., 2000), fungal NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (Verho et al., 2003), non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Bro et al., 2006), cytoplasmic transhydrogenase (Nissen et al., 2001), malic enzyme (Moreira dos Santos et al., 2004) and NADH kinase (Hou et al., 2009b).

In contrast to the above-mentioned approaches, water-forming NADH oxidase (EC 1.6.99.3) oxidizes NADH to NAD^+ using molecular oxygen as an electron acceptor and produces water. Genes coding for NADH oxidase have been isolated mainly from facultative anaerobic bacteria. Among them, *noxE* from *Lactococcus lactis* (Heux et al., 2006) and *nox* from *Streptococcus pneumoniae* (Hou et al., 2009a; Vemuri et al., 2007) have been functionally expressed in *S. cerevisiae*. Expression of the NADH oxidases led to a decrease in intracellular NADH concentration and NADH/NAD^+ ratio (Heux et al., 2006; Hou et al., 2009a; Vemuri et al., 2007). This reduced NADH availability shifted fermentation products from ethanol, glycerol, succinate and hydroxyglutarate into more oxidized metabolites, such as acetaldehyde, acetate and acetoin (Heux et al., 2006).

In this study, *L. lactis* NADH oxidase was expressed to reduce glycerol while increasing 2,3-BD production in the 2,3-BD-producing Pdc-deficient *S. cerevisiae*. The five engineered *S. cerevisiae* strains with different expression levels of NADH oxidase were constructed by employing plasmids with different copy numbers and promoters with various strengths. Batch fermentations of the engineered *S. cerevisiae* were carried out to determine the effect of NADH oxidase on 2,3-BD fermentation. Alteration of redox status in the engineered *S. cerevisiae* by *L. lactis* NADH oxidase changed the metabolic profiles on 2,3-BD fermentation.

2. Methods

2.1. Construction of plasmids

Strains and plasmids used in this study are summarized in Table 1. The primers used for cloning of *S. cerevisiae* promoters and NADH oxidase gene from *L. lactis* subsp. *cremoris* MG1363 are listed in Table 2. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) was used for gene cloning and manipulation. *E. coli* transformants were grown in Lysogeny Broth (LB) medium with 50 μg/mL of ampicillin. To construct expression plasmids with different promoters, upstream regions of *S. cerevisiae CYC1* (289 bp) and *GPD2* (1144 bp) were amplified by PCR from genomic DNA of *S. cerevisiae* D452-2 using the primers in Table 2, and *S. cerevisiae TDH3* promoter (655 bp) was purified from p426GPD plasmids after *SacI* and *XbaI* treatment. The promoter DNA fragments were ligated into appropriate restriction sites in pRS406 and pRS426 plasmids. In order to construct *L. lactis* NADH oxidase expression plasmids, the *L. lactis noxE* gene was amplified by PCR with F_nox and R_XhoI_nox for plasmids containing *GPD2* and *TDH3* promoter, and F_nox and R_SalI_nox for p426CYC1 plasmid. The amplified DNA fragments were ligated into the corresponding restriction sites in Table 2.

2.2. Yeast transformation and construction of recombinant S. cerevisiae strains

The Pdc-deficient *S. cerevisiae* SOS2 (Kim et al., 2013) was used as a host strain for the construction of engineered *S. cerevisiae* strains listed in Table 1. The *S. cerevisiae* SOS5 was constructed by deleting the *PDC6* gene in the SOS2 strain by *URA3* marker recycling method as mentioned previously (Kim et al., 2013) using the primers described in Table 2. Transformation of plasmids for introducing the 2,3-BD biosynthetic pathway, and NADH oxidase was performed using a spheroplast transformation kit (BIO 101, Vista, CA). To select transformants, *S. cerevisiae* strains were routinely cultivated aerobically at 30 °C in YNB medium (6.7 g/L yeast

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Description	References
Strains		
Lactococcus lactis subsp. cremoris MG1363	Source for *noxE*	In this study
D452-2	*S. cerevisiae* MATα, leu2, his3, ura3, can1	Nikawa et al. (1991)
SOS2	D452-2, Δpdc1, Δpdc5	Kim et al. (2013)
SOS5	D452-2, Δpdc1, Δpdc5, Δpdc6	In this study
BD5	SOS5, p423_alsSalsD, p425_BDH1	In this study
BD5_Con	BD5, p426GPD	In this study
BD5_G1nox	BD5, *ura3::URA3* p426GPD2_nox	In this study
BD5_T1nox	BD5, *ura3::URA3* p426TDH3_nox	In this study
BD5_C2nox	BD5, p426CYC1_nox	In this study
BD5_G2nox	BD5, p426GPD2_nox	In this study
BD5_T2nox	BD5, p426TDH3_nox	In this study
Plasmids		
pRS406	URA3	Mumberg et al. (1995)
pRS426	*URA3* 2 μm origin	Mumberg et al. (1995)
p426GPD	*URA3* 2 μm origin $TDH3_{prom}$ $CYC1_{term}$	Mumberg et al. (1995)
p406GPD2	pRS406, $GPD2_{prom}$ $CYC1_{term}$	In this study
p406TDH3	pRS406, $TDH3_{prom}$ $CYC1_{term}$	In this study
p426CYC1	pRS426, $CYC1_{prom}$ $CYC1_{term}$	In this study
p426GPD2	pRS426, $GPD2_{prom}$ $CYC1_{term}$	In this study
p423_alsSalsD	*HIS3* 2 μm origin $TDH3_{prom}$-*alsS*-$CYC1_{term}$ $TDH3_{prom}$-*alsD*-$CYC1_{term}$	Kim et al. (2014)
p425_BDH1	*LEU2* 2 μm origin $TDH3_{prom}$-*BDH1*-$CYC1_{term}$	Kim et al. (2013)
p406GPD2_nox	pRS406, $GPD2_{prom}$-*noxE*-$CYC1_{term}$	In this study
p406TDH3_nox	pRS406, $TDH3_{prom}$-*noxE*-$CYC1_{term}$	In this study
p426CYC1_nox	pRS426, $CYC1_{prom}$-*noxE*-$CYC1_{term}$	In this study
p426GPD2_nox	pRS426, $GPD2_{prom}$-*noxE*-$CYC1_{term}$	In this study
p426TDH3_nox	pRS426, $TDH3_{prom}$-*noxE*-$CYC1_{term}$	In this study

Table 2
Primers used in this study. Bold and capital characters are restriction enzyme sites.

Primers	Restriction site	Sequence
Deletion of PDC6		
F_d_PDC6-1	***Xba*I**	GC**TCTAGA**tcttaatgtttagcagctc
R_d_PDC6-2		gattaagatagaatggcttc
F_d_PDC6-3	***Asc*I**	gaagccattctatcttaatc**GGCGCGCC** tactacgttatcgccgtg aattacgcaattcgcatgttacgattcgg taatctccgaa
R_d_PDC6-4	***Xba*I**	GC**TCTAGA**tgtatttatttgtctgattg atttatgtattcaaactgtggggtaa taactgatataatta
R_d_PDC6-Check		gctgaacaacagtctctc
Cloning of S. cerevisiae promoters		
F_CYC1P	***Sac*I**	c**GAGCTC**atttggcgagcgttg
R_CYC1P	***Bam*HI**	cgc**GGATCC**ttagtgtgtgtatttgtgtttgc
F_GPD2P	***Sac*I**	c**GAGCTC**caaaaacgacatatctattatagtg
R_GPD2P	***Bam*HI**	cgc**GGATCC**ctttgagtgcagttgtgttt
Cloning of L. lactis noxE		
F_nox	***Bam*HI**	cgc**GGATCC**aaaatgaaaatcgtagttatcggta
R_XhoI_nox	***Xho*I**	ccg**CTCGAG**tttatttggcattcaaagct
R_SalI_nox	***Sal*I**	acgc**GTCGAC**tttatttggcattcaaagct

nitrogen base and appropriate nucleotides and amino acid). An ethanol concentration of 20 g/L was used as a carbon source for culturing the SOS5 strain and 20 g/L glucose and 1 g/L ethanol for culturing the other strains. The *B. subtilis alsS*, *alsD* and *S. cerevisiae BDH1* genes were expressed under the constitutive *S. cerevisiae TDH3* promoter. The *S. cerevisiae* BD5 strain was constructed by transformation of p423_alsS_alsD and p425_BDH1 plasmids into the SOS5 strain. The *L. lactis noxE* gene was expressed under different native promoters (*TDH3*, *CYC1*, and *GPD2*) and copy number (single and muticopy). The *L. lactis noxE* expression plasmids in Table 1 were introduced into the BD5 strain and five engineered *S. cerevisiae* strains expressing different levels of NADH oxidase were constructed. Integrative *L. lactis noxE* expression plasmids (p406GPD2_nox, p406TDH3_nox) were digested with *StuI* before use and integrated into the *URA3* locus. The resulting recombinant *S. cerevisiae* strains are listed in Table 1.

2.3. Fermentation conditions

All cultures were carried out at 30 °C. Pre-cultures of yeast cells were conducted aerobically in 250 mL baffled flasks. Main flask batch cultures were conducted under oxygen-limited conditions in 250 mL flasks at 80 rpm. In order to prepare inoculums, engineered *S. cerevisiae* cells were cultivated during for 48 h in 5 mL YNB medium containing 20 g/L glucose and 1 g/L ethanol. The grown cells were transferred to 100 mL YNB medium containing 20 g/L glucose and 0.5 g/L ethanol. After 24 h cultivation, the mid-exponential growing cells (OD_{600} < 3) were harvested and washed twice with double-distilled water (DDW). Cells were inoculated into the main culture at the initial concentration of 0.20 g DCW/L. The main culture YNB medium contains 80 g/L glucose and 50 mM potassium phthalate at pH 5.5 adjusted by NaOH. Ethanol 0.5 g/L was added if necessary.

Fermentations with bioreactor were carried out in 500 ml YNB medium containing 90 g/L glucose and 0.7 g/L ethanol using 1 L-bench-top fermentor (Fermentec, Korea) at 30 °C. The medium pH was maintained at 5.5 with 5 N NaOH solution and dissolved oxygen (DO) levels were monitored with O_2 sensor (Mettler Toledo, Switzerland). The culture medium was agitated at 200 rpm and aerated with air flow rate of 0.3 vvm. The grown cells were prepared as flask culture and inoculated at the initial concentration of 0.10 g DCW/L.

2.4. Analysis of dry cell weight and metabolites

Cell growth was monitored by optical density at 600 nm (OD_{600}) using a spectrophotometer (UV-1601, Shimadzu, Japan). Dry cell weight (DCW) was calculated using a pre-determined factor of 0.20 g dry cell/L/OD_{600}. Glucose, glycerol, acetoin, pyruvate, and 2,3-BD were analyzed by a high-performance liquid chromatography (1100 series, Agilent, CO) equipped with a Rezex ROA-organic acid column (Phenomenex, CA). Pyruvate was detected by a UV detector at 214 nm and the other metabolites were detected by a refractive index (RI) detector. NADH and NAD^+ concentrations were measured using EnzyChromTM NAD^+/NADH Assay Kit (ECND-100, BioAssay Systems, CA). About 4×10^7 cells in flask batch cultivation at 48 h were harvested and used to determine NADH and NAD^+ concentrations. NADH and NAD^+ concentration assays were conducted following manufacturer-provided methods (BioAssay Systems, CA).

2.5. In vitro enzymatic assay of NADH oxidase

To prepare crude extracts, about 1×10^9 mid-exponential phase cells grown on the YNB medium with 80 g/L glucose and 0.5 g/L ethanol in a flask culture were harvested and washed twice with DDW. Protease inhibitor (Roche, Switzerland) was added and the harvested cells were lysed with Yeast Protein Extraction Reagent (Thermo Scientific, MA). After centrifugation for 20 min at 12,000 rpm and 4 °C, the supernatants were used to determine the NADH oxidase activity within 3 h and diluted with DDW as necessary. The NADH oxidase activity assays were performed at 30 °C with the reaction mixture containing 50 mM potassium

phosphate buffer (pH 7.0), 0.4 mM NADH, and 0.3 mM EDTA with some modifications based on a previous report (De Felipe et al., 1998). The reactions were initiated by adding a crude extract, and a decrease of absorbance at 340 nm was measured. One unit of activity was defined as the amount of enzyme oxidizing 1 μmol NADH per minute at the corresponding reaction conditions. The protein concentration of crude extracts was determined by the Bradford method (Bradford, 1976).

3. Results and discussion

3.1. Introduction of the 2,3-BD biosynthetic pathway and the expression of L. lactis noxE in Pdc-deficient S. cerevisiae

To construct 2,3-BD-producing *S. cerevisiae*, *B. subtilis* acetolactate synthase (*alsS*), acetolactate decarboxylase (*alsD*) and *S. cerevisiae* 2,3-butanediol dehydrogenase 1 (*BDH1*) were overexpressed in the Pdc-deficient *S. cerevisiae* SOS5 (Table 1). In order to vary expression levels of *L. lactis noxE*, three endogenous promoters (*CYC1*, *GPD2*, and *TDH3*) and two plasmids with different copy numbers (integrative and episomal) were combined as shown in Table 1. The rationale of selecting the three promoters for altering expression levels of *noxE* are as follow. Generally, the *S. cerevisiae CYC1* promoter is regarded as a weak promoter whereas the *TDH3* promoter is regarded as a strong promoter (Mumberg et al., 1995). The *S. cerevisiae GPD2* promoter is known to be induced by anaerobiosis (Eriksson et al., 1995).

The control and *L. lactis noxE* expressing plasmids (p426GPD, p406GPD2_nox, p406_TDH3_nox, p426CYC1_nox, p426GPD2_nox, p426_TDH3_nox) were introduced to a Pdc-deficient *S. cerevisiae* expressing the 2,3-BD-producing pathway (the BD5 strain), and yielded the control and five engineered 2,3-BD-producing *S. cerevisiae* strains with different expression levels of *L. lactis noxE* (Table 1).

3.2. Differential NADH oxidase activities influenced intracellular NADH and NAD^+ concentration in yeast

To determine expression levels of NADH oxidase in engineered *S. cerevisiae* strains expressing *noxE* differentially, NADH oxidase activities of the control and five *noxE* expressing strains were measured. As shown in Fig. 1, enzymatic activities of NADH oxidase in the five engineered *S. cerevisiae* strains were correlated with different copy numbers and promoters. While the control strain (BD5_Con) showed NADH oxidase activity of 4.8 mU/mg protein, we can speculate that this background activity might be observed from other NADH-consuming reactions using metabolites in the crude extract such as yeast native NADH dehydrogenase NDE1/NDE2. The engineered strain BD5_G1nox with a single copy integration of *noxE* under the control of the *GPD2* promoter showed 11.2 mU/mg protein which is the smallest value of NADH oxidase activity among the five *noxE* expressing strains. On the other hand, the BD5_T2nox strain with *noxE* in a multi-copy plasmid under the control of the strong constitutive promoter *TDH3*, showed an almost 900-fold higher NADH oxidase activity of 9.153 U/mg protein than the BD5_G1nox strain (Fig. 1).

Overexpression of NADH oxidase in *S. cerevisiae* is known to decrease the intracellular NADH/NAD^+ ratio substantially (Heux et al., 2006; Vemuri et al., 2007). In order to confirm whether the expression levels of NADH oxidase could affect intracellular NADH/NAD^+ ratios in the five engineered strains, cells grown in the mid-exponential phase on minimal medium were used to measure the intracellular NADH and NAD^+ concentrations. Intracellular concentrations of NADH and NAD^+ for the control and five *noxE* expressing strains are summarized in Table S1. The NADH/NAD^+ ratios decreased by the expression of NADH oxidase. As a result, the NADH/NAD^+ ratio of the BD5_T2nox strain was much lower (0.14 vs. 0.31) than that of the BD5_Con strain. The NADH/NAD^+ ratio of the BD5_C2nox strain, which showed a moderate expression level of NADH oxidase, was 0.24. Additionally, the NADH/NAD^+ ratios of BD5_T1nox and BD5_G2nox were similar (0.13 and 0.16) to the BD5_T2nox strain, although *in vitro* NADH oxidase activities of BD5_T2nox were 19- and 15-folds higher than the BD5_T1nox and BD5_G2nox strains. The intracellular NADH/NAD^+ ratios depend on *in vivo* activities of NADH oxidase in NADH oxidase expressing strains, but the activities of NADH oxidase might be limited by the dissolved molecular oxygen levels in medium in contrast with *in vitro* enzymatic activity assay. The NADH/NAD^+ ratios in the presence of ethanol which is necessary to enhance the growth of a Pdc-deficient mutant, were also measured to check if the supplementation of ethanol can alter the ratios of NADH/NAD^+. The NADH/NAD^+ ratios in the engineered *S. cerevisiae* strains did not change even after supplementation of ethanol into the medium (Fig. S1).

3.3. Production of 2,3-BD by the Pdc-deficient S. cerevisiae expressing 2,3-BD biosynthetic pathway and NADH oxidase in minimal medium with glucose as a sole carbon source

To evaluate the effect of overexpression of NADH oxidase on 2,3-BD fermentation by engineered *S. cerevisiae*, oxygen-limited flask cultures were carried out in minimal medium with 90 g/L glucose as a sole carbon source (Fig. S2). 2,3-BD and glycerol were major products and acetoin was a minor product by the BD5_Con

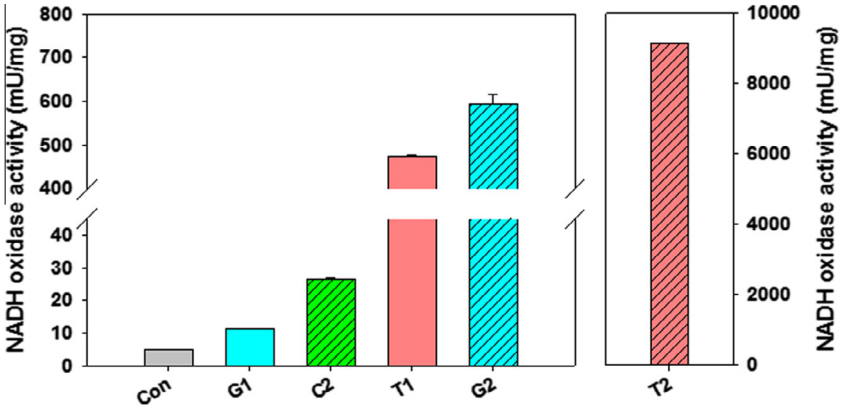

Fig. 1. The specific activities of NADH oxidase in the control and five engineered strains expressing *noxE* differentially. BD5_Con (Con), BD5_G1nox (G1), BD5_T1nox (T1), BD5_C2nox (C2), BD5_G2nox (G2), and BD5_T2nox (T2) during mid-exponential phase of oxygen-limited batch fermentation.

strain (0.304 g 2,3-BD/g glucose, 0.286 g glycerol/g glucose and 0.012 g acetoin/g glucose). On the other hand, fermentation products by the BD5_T2nox strain were quite different from the BD5_Con strain. In contrast with the BD5_Con strain, the BD5_T2nox strain produced substantial amounts of acetoin (0.223 g acetoin/g glucose) with reduced amounts of 2,3-BD and glycerol (0.165 g 2,3-BD/g glucose and 0.132 g glycerol/g glucose). These results suggest that metabolic fluxes from glucose toward 2,3-BD and glycerol production were shifted toward acetoin production by the strong expression of NADH oxidase in the BD5_T2nox strain. Decreased cytosolic NADH/NAD^+ ratio in the BD5_T2nox strain (Fig. S1) could not redirect carbon flux from glycerol to 2,3-BD but rather accelerated acetoin formation from glucose. The large reduction in the NADH/NAD^+ ratio in *S. cerevisiae* strains by expression of NADH oxidase during growth on glucose resulted in decreased biomass yield and redistributed metabolic fluxes (Heux et al., 2006; Vemuri et al., 2007). Similarly, in the minimal medium with glucose as a sole carbon source, the growth and fermentation capacity of 2,3-BD-producing Pdc-deficient *S. cerevisiae* were substantially reduced by expression of NADH oxidase (Fig. S2B). While the control strain of BD5_Con grew to 0.85 g DCW/L and consumed 26.2 g/L glucose in 70 h cultivation, the BD5_T2nox strain expressing *noxE* with a strong promoter showed slower growth and glucose consumption (0.25 g DCW/L and 11.2 g/L glucose) than the BD5_Con strain. The inhibitory effects by acetaldehyde in *noxE*-expressing strains (Heux et al., 2006) might not be a reason for slow growth and a poor fermentation capacity of the engineered strains because acetaldehyde cannot be synthesized in Pdc-deficient *S. cerevisiae* strains. The higher acetoin accumulation and lower growth of the BD5_T2nox strain than the BD5_Con strain on glucose might result from excess oxidation of cytosolic NADH by NADH oxidase. The activity of NADH oxidase might be too high to maintain the redox state because of slow growth and glucose uptake rates of Pdc-deficient *S. cerevisiae* on glucose.

3.4. The introduction of 2,3-BD biosynthetic pathway allows the growth of Pdc-deficient S. cerevisiae on glucose as a sole carbon source

Pdc-deficient *S. cerevisiae* strains have been constructed as platform strains for further metabolic engineering and production of pyruvate which can serve as an effective precursor for many chemicals (van Maris et al., 2004). However, the Pdc-deficient *S. cerevisiae* strains exhibited growth defects hindering the usage of them as platform strains for industrial applications. Pdc-deficient *S. cerevisiae* strains require the supplementation of C_2-compounds such as ethanol or acetate to synthesize the cytosolic acetyl-CoA to grow on glucose as a sole carbon source (Flikweert et al., 1996). Furthermore, even in the presence of C_2-compounds, the insufficient regeneration of cytosolic NAD^+ resulted in slow cell growth and glucose consumption rates (Ishida et al., 2006). As a result, the Pdc-deficient *S. cerevisiae* strains are unable to grow in batch cultures on defined media with glucose as a sole carbon source (Flikweert et al., 1996). As recently reported, the mutation of *MTH1* allows the Pdc-deficient *S. cerevisiae* strains to overcome the growth defects on glucose (Oud et al., 2012). Mth1p regulates glucose transporters by inactivating the transcriptional regulator Rgt1 (Moriya and Johnston, 2004). Reduced glucose influx by the *MTH1* mutation in Pdc-deficient *S. cerevisiae* strains partially alleviated the demand for C_2-compounds and retardation of cell growth on glucose (Oud et al., 2012). In this study, however, the BD5_Con strain with the wild type *MTH1* could grow on a synthetic glucose medium despite of the low growth rate (Fig. S2A), and the cell growth can be maintained even after serial cultivations (data not shown). It seems that the introduction of the 2,3-BD biosynthetic pathway could alleviate growth defects on glucose in the Pdc-deficient *S. cerevisiae* strain through regeneration of NAD^+ (Nan et al., 2014) and supplementation of C_2-compounds. Because the 2,3-BD biosynthetic pathway consumes 1 mole of NADH, the redox state in the 2,3-BD-producing strain could be more balanced than the Pdc-deficient strains without the 2,3-BD pathway. Although the C_2-independent property of the Pdc-deficient *S. cerevisiae* with the 2,3-BD biosynthetic pathway is still unknown, there are several possibilities of supplying C_2-compounds by (i) direct cleavage of acetoin into acetaldehyde and acetate (López et al., 1975), and (ii) non-specific decarboxylation of pyruvate to acetaldehyde by *B. subtilis* acetolactate synthase (*alsS*) (Atsumi et al., 2009).

3.5. Effect of different levels of NADH oxidase on 2,3-BD fermentation in minimal media with glucose and ethanol

Although the 2,3-BD-producing Pdc-deficient *S. cerevisiae* strain could grow in minimal medium with glucose as a sole carbon source, cell growth and glucose consumption rates were substantially lower than those of wild type *S. cerevisiae*. The synthesis of cytosolic acetyl-CoA in 2,3-BD-producing Pdc-deficient *S. cerevisiae* strains was speculated as a rate-limiting step in cell growth and 2,3-BD production. Therefore, in order to improve growth of the 2,3-BD-producing Pdc-deficient *S. cerevisiae*, 0.5 g/L of ethanol was supplemented as a C_2-compound for synthesizing cytosolic acetyl-CoA in fermentation experiments. The fermentation profiles of the control strain (BD5_Con) and NADH oxidase-expressing strain (BD5_T2nox) are shown in Fig. 2. The fermentation profiles of the other engineered *S. cerevisiae* strains are shown in Fig. S3.

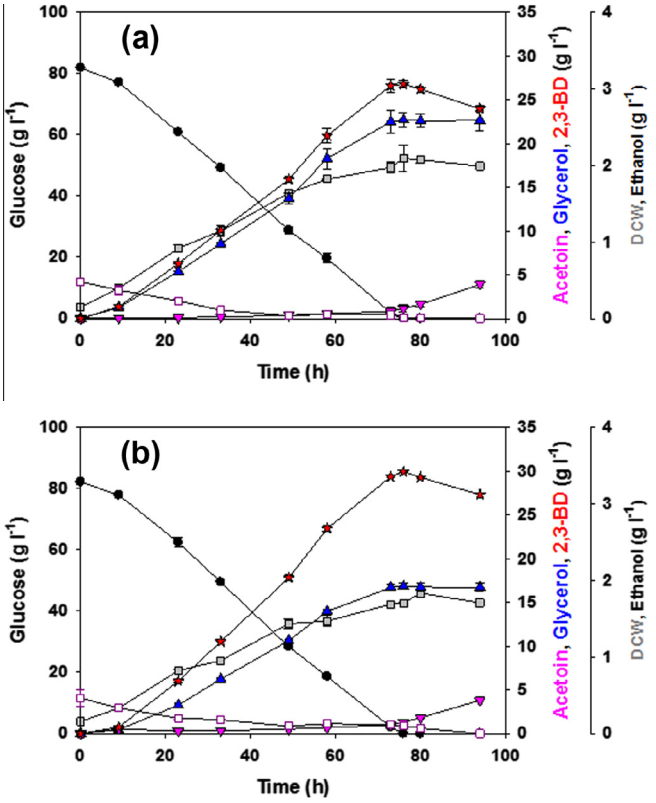

Fig. 2. Representative fermentation profiles of (a) BD5_Con and (b) BD5_T2nox among engineered 2,3-BD producing *S. cerevisiae* strains in oxygen-limited batch cultivation using a minimal medium containing 80 g/L glucose and 0.5 g/L ethanol. Symbols: glucose (filled circle), dry cell weight (grey square), glycerol (blue triangle), 2,3-BD (red star), acetoin (pink inverted triangle), and ethanol (open square). Results are the averages of duplicate experiments with error bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

All strains were able to consume 80 g/L glucose in 76 h and the supplemented 0.5 g/L ethanol remained in the medium until all glucose was depleted. Values of maximum DCW, product yields, and glucose uptake rates by engineered strains expressing different levels of NADH oxidase are compared in Table 3. When glucose was used as a sole carbon source in fermentation experiments, BD5_T2nox showed substantially reduced cell growth and glucose uptake rates. Once ethanol is supplemented, however, the growth and glucose consumption rates of the BD5_T2nox strain largely increased. By supplementing with a C_2-compound, the redox state in the cytosol was maintained due to the overall improved cellular metabolism. Although the final dry cell weights (DCW) of all NADH oxidase expressing strains were 7.7–25.8% lower than that of the BD5_Con strain, volumetric glucose uptake rates were similar in all tested strains (Table 3). As such, specific glucose uptake rates (g 2,3-BD/L h g DCW) increased for the strains expressing NADH oxidase. It is assumed that NAD^+ which is necessary for continued flux through glycolysis, could be efficiently re-generated by expression of NADH oxidase. Further studies to identify the improvement of the glycolytic pathway by expression of NADH oxidase in Pdc-deficient *S. cerevisiae* might be required.

When 0.5 g/L of ethanol was supplemented, 2,3-BD was predominantly produced rather than acetoin and glycerol was a major by-product for all tested strains. Introduction of NADH oxidase decreased the NADH/NAD^+ ratio, and influenced the product formation pattern of 2,3-BD fermentation by engineered yeast (Fig. 2). Glycerol mainly serves as a redox sink in *S. cerevisiae* by consuming the surplus cytosolic NADH under oxygen-limited conditions (Ansell et al., 1997). Alleviation of excess cytosolic NADH by NADH oxidase could result in reduced glycerol production and increased 2,3-BD production in the 2,3-BD-producing Pdc-deficient *S. cerevisiae* strains. The NADH/NAD^+ ratio of the BD5_T2nox strain (0.14) was substantially lower than that of the BD5_Con strain (0.31) during batch cultivation. As a result, the glycerol yield of the BD5_T2nox strain was 25.1% lower than that of the BD5_Con strain, and the 2,3-BD yield of the BD5_T2nox strain increased 10.7% compared to the BD5_Con strain. These results indicated that glycerol and 2,3-BD production can be controlled by an intracellular NADH/NAD^+ ratio. Through the expression of NADH oxidase, glucose flux could be rerouted to 2,3-BD production instead of glycerol production when a small amount of ethanol was supplemented.

During the early exponential phase (<9 h), acetoin was rapidly produced in NADH oxidase expressing strains (Fig. S4). In the BD5_T2nox strain, the highest acetoin (0.53 g/L) was produced among the engineered strains, which was 9-fold higher than that of the BD5_Con strain (0.06 g/L). However, produced acetoin was re-assimilated between 9 h and 24 h (Fig. S4). Despite the large difference of produced acetoin during the early exponential phase, the final yields of acetoin were similar within all tested strains and occupied only 1% of glucose consumed (0.009–0.012 g acetoin/g glucose) (Table 3). The acetoin can be produced from a reverse reaction of 2,3-BD by 2,3-butanediol dehydrogenase (*BDH1*) besides a decarboxylation of α-acetolactate by acetolactate decarboxylase (*alsD*). The interconversion of acetoin and 2,3-BD by 2,3-butanediol dehydrogenase was affected by the redox state in engineered *S. cerevisiae* strain. In the early exponential phase (<9 h), NADH oxidase can be fully activated because there was enough dissolved molecular oxygen in the medium. During this phase, NADH oxidase could efficiently oxidize the cytosolic NADH and acetoin was accumulated mainly proportional to the NADH oxidase activity. After dissolved molecular oxygen was depleted and oxygen-limited conditions were created, metabolic reactions were changed from acetoin to 2,3-BD production because of limited NADH oxidase activity. When all glucose was depleted in the medium (>76 h), substantial amounts of acetoin were generated along with decreases of 2,3-BD (Fig. 2). After glucose depleted (>76 h), the generation of NADH by glycolysis and 2,3-BD production was stopped. At this time, 2,3-BD might be oxidized to acetoin by a reverse reaction of 2,3-butanediol dehydrogenase due to the elevated cytosolic NAD^+ level.

Although the expression of NADH oxidase could reduce glycerol and increase 2,3-BD production (Table 3), the effects on 2,3-BD fermentation profiles were similar in the four strains with activity above 20 mU/mg protein (BD5_T1nox, BD5_C2nox, BD5_G2nox and BD5_T2nox). Moreover, glycerol yields of NADH oxidase expressing strains were still exceeded 0.2 g glycerol/g glucose. This means that regeneration of excess cytosolic NADH still largely depends on the glycerol production. The shortage of available molecular oxygen in oxygen-limited conditions could be one of the reasons for limited effects of NADH oxidase.

The effect of NADH oxidase on product profiles during 2,3-BD fermentation were examined during batch fermentations in bioreactor with BD5_Con strain and BD5_T2nox strain which exhibited drastic phenotype changes in previous experiments (Fig. 3). As previously reported (Heux et al., 2006), the BD5_T2nox strain expressing the NADH oxidase showed a higher oxygen demand than the control strain. The dissolved oxygen was depleted completely in 35 h by the BD5_T2nox strain while the dissolved oxygen level was maintained over 25% of the saturation value in the fermentation medium by the BD5_Con strain (Fig. S5). As excess cytosolic NADH could be oxidized by NADH oxidase with molecular oxygen as a substrate in the BD5_T2nox strain, glycerol yield of the BD5_T2nox strain (0.069 g glycerol/g glucose) was reduced as compared to BD5_Con strain (0.199 g glycerol/g glucose). Therefore, the carbon flux was redirected from glycerol into 2,3-BD production by expression of NADH oxidase. The 2,3-BD yield of the BD5_T2nox strain (0.359 g 2,3-BD/g glucose) was 23.8% higher than that of the BD5_Con strain (0.290 g 2,3-BD/g glucose). In contrast to flask batch cultivation, acetoin yield increased in the BD5_T2nox strain (0.052 g acetoin/g glucose) as compared with the BD5_Con strain (0.018 g acetoin/g glucose). It might result

Table 3
Fermentation parameters of engineered *S. cerevisiae* strains in oxygen-limited batch fermentation with a minimal medium containing 80 g/L glucose and 0.5 g/L ethanol.

Parameter[a]	Value (mean ± standard deviation)					
	BD5_Con	BD5_G1nox	BD5_C2nox	BD5_T1nox	BD5_G2nox	BD5_T2nox
DCW (g/L)	2.09 ± 0.10	1.93 ± 0.07	1.74 ± 0.11	1.55 ± 0.09	1.68 ± 0.04	1.69 ± 0.03
$Y_{glycerol}$ (g/g)	0.278 ± 0.011	0.266 ± 0.010	0.229 ± 0.001	0.231 ± 0.005	0.216 ± 0.009	0.209 ± 0.004
$Y_{2,3\text{-BD}}$ (g/g)	0.332 ± 0.000	0.338 ± 0.001	0.359 ± 0.000	0.359 ± 0.001	0.364 ± 0.003	0.367 ± 0.002
$Y_{acetoin}$ (g/g)	0.010 ± 0.001	0.009 ± 0.001	0.012 ± 0.001	0.011 ± 0.001	0.010 ± 0.000	0.012 ± 0.001
$V_{glucose}$ (g/L h^{-1})	1.10 ± 0.03	1.11 ± 0.02	1.06 ± 0.01	1.13 ± 0.00	1.07 ± 0.02	1.10 ± 0.00
$P_{2,3\text{-BD}}$ (g/L h^{-1})	0.364 ± 0.011	0.374 ± 0.008	0.381 ± 0.003	0.404 ± 0.002	0.391 ± 0.006	0.403 ± 0.002

[a] DCW, maximum DCW (g DCW/L); $Y_{glycerol}$, glycerol yield (g glycerol/g glucose); $Y_{2,3\text{-BD}}$, 2,3-butanediol yield (g 2,3-butanediol/g glucose); $Y_{acetoin}$, acetoin yield (g acetoin/g glucose); $V_{glucose}$, volumetric glucose consumption rate (g glucose/L h^{-1}); $P_{2,3\text{-BD}}$, 2,3-butanediol productivity (g 2,3-butanediol/L h^{-1}).

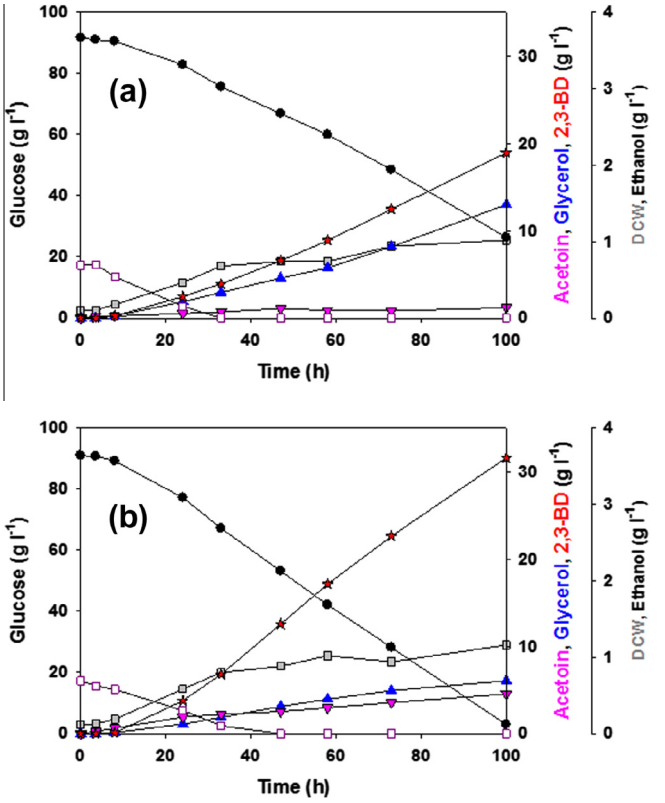

Fig. 3. Fermentation profiles of (a) BD5_Con and (b) BD5_T2nox in minimal medium with bioreactor. Symbols: glucose (filled circle), dry cell weight (grey square), glycerol (blue triangle), 2,3-BD (red star), acetoin (pink inverted triangle), and ethanol (open square). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from excessive reaction of NADH oxidase of the BD5_T2nox strain in a bioreactor. Controlling and optimizing the oxygen levels in culture medium would be necessary for efficient production of 2,3-BD by *noxE* expressing strains. In addition, strain improvement through deletion of the glycerol 3-phosphate dehydrogenase gene, or introduction of additional cofactor engineering systems, could be applied to further increasing 2,3-BD yield and decreasing by-products. Water-forming NADH oxidase uses molecular oxygen as a substrate to oxidize NADH and does not require any metabolic intermediates for reaction unlike other cofactor engineering processes. Thus, the reaction of NADH oxidase could be simply regulated by combined with protein expression levels and oxygen supply. The simplified control of intracellular NADH/NAD^+ levels through the expression of NADH oxidase and oxygen supplying could make 2,3-BD production process feasible in large scale fermentations. In addition, the expression and control of NADH oxidase could be an efficient strategy for cofactor engineering to produce fine chemicals which require effective regeneration of cofactors.

4. Conclusions

In this study, the *B. subtilis* 2,3-BD biosynthetic pathway enzymes, *S. cerevisiae* 2,3-butanediol dehydrogenase, and *L. lactis* NADH oxidase were expressed to efficiently produce 2,3-BD in Pdc-deficient *S. cerevisiae*. The introduction of the 2,3-BD biosynthetic pathway in Pdc-deficient *S. cerevisiae* enabled the strain to grow in synthetic glucose medium without C_2-compounds. In addition, the introduction of NADH oxidase with supplementation of ethanol as a C_2-compound successfully led to enhanced growth and glucose consumption along with reduced glycerol production and increased 2,3-BD production in Pdc-deficient *S. cerevisiae*.

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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.2015.02.077.

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