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

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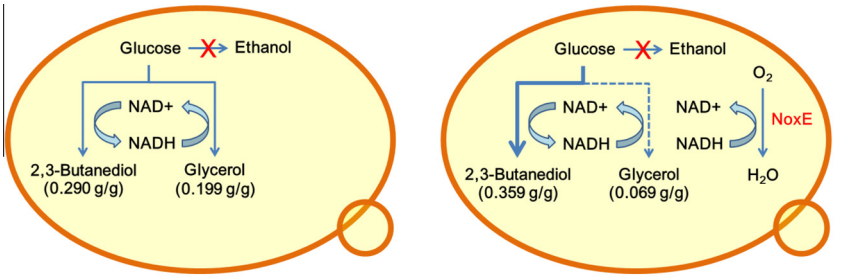
HIGHLIGHTS

- *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