농학박사학위논문

효모의 보효소 및 대사경로 재설계를 통한 2,3-butanediol 의 생산

Cofactor and pathway engineering of *Saccharomyces cerevisiae* for enhanced production of 2,3-butanediol

2016 년 2 월

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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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February 2016

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MAJOR IN FOOD BIOTECHNOLOGY

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

GRADUATE SCHOOL

SEOUL NATIONAL UNIVERSITY

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Advisor: Professor Jin-Ho Seo

A dissertation submitted in partial fulfillment of

The requirements for the degree of

DOCTOR OF PHILOSOPHY

to the Faculty of Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

Jin-Woo Kim

February 2016

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2015 년 12 월

서울대학교 농생명공학부 식품생명공학전공
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2015 년 12 월

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ABSTRACT

2,3-Butanediol (2,3-BD) is a versatile chemical that has various applications to chemical industries. For efficient production of 2,3-BD by microbial fermentation, 2,3-BD biosynthetic enzymes including acetolactate synthase (AlsS), acetolactate decarboxylase (AlsD), and 2,3-butanediol dehydrogenase (Bdh) were introduced into pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae*. The engineered Pdc-deficient *S. cerevisiae* (BD5) strain produced 2,3-BD without ethanol formation. However, two drawbacks of 'cofactor imbalance' and 'C_2-auxotropy' in Pdc-deficient *S. cerevisiae* strain hinder a development of efficient 2,3-BD production system for industrial use. The cofactor imbalance induced by an accumulation of cytosolic NADH produced a large amount of glycerol as a byproduct and reduced 2,3-BD yield. In addition, the C_2-auxotropy which means a lack of cytosolic acetyl-CoA synthesis lowers cell growth rate and 2,3-BD productivity. In this study, the cofactor imbalance and the C_2-auxotropy are overcome to increase 2,3-BD production by engineering of metabolic pathways in Pdc-deficient strains which are associated with NADH metabolism and acetyl-CoA biosynthesis. To reduce cofactor imbalance, an NADH-oxidizing pathway by *Lactococcus lactis* NADH oxidase (*noxE*) was

introduced. The expression of NADH oxidase reduced NADH/NAD^+ ratio, and changed carbon flux from glycerol to 2,3-BD. To alleviate C_2-auxotropy, supplementation of a trace amount of C_2-compounds such as ethanol was necessary. Fine-tuned expression of *PDC* by the combination of *PDC* gene source, promoter, and copy number overcame C_2-auxotropy without supplementation of C_2-compounds and led to enhanced cell growth rate and 2,3-BD productivity. The BD5_Ctnox strain co-expressing *noxE* and fine-tuned *PDC* produced 154.3 g/L of 2,3-BD with 1.98 g/L/h of productivity and 0.404 $g_{2,3\text{-}BD}/g_{Glucose}$ of 2,3-BD yield in the fed-batch fermentation. Additionally, to further increase 2,3-BD yield by elimination of glycerol production, the glycerol synthetic genes of glycerol-3-phosphate dehydrogenase (*GPD*) were deleted. Since NADH oxidase oxidizes an additional NADH by using molecular oxygen as an electron acceptor, the *gpd* null mutant strains could survive in spite of a severe cofactor imbalance induced by dual deletion of *gpd* and *pdc* in *S. cerevisiae*. The *gpdΔ* strain resulted in complete elimination of glycerol formation. From the fed-batch fermentation with the engineered strain co-expressed *noxE* and fine-tuned *PDC* and deleted *GPD* (BD5_Ctnox_dGPD1dGPD2), 108.6 g/L 2,3-BD was produced in 76 h cultivation. The yield of 2,3-

BD (0.462 $g_{2,3\text{-BD}}/g_{Glucose}$) was corresponded to 92.4% of theoretical yield. The strain engineering in this study successfully improved 2,3-BD production in *S. cerevisiae*. The 2,3-BD production performance of the engineered strains was superior to that of the other 2,3-BD production yeasts. Especially, 2,3-BD titer of 154.3 g/L is the highest value among other microbial production studies. The strategies of cofactor engineering by NADH oxidase and pathway engineering with fine-tuned Pdc expression could be useful for other research on chemical production using Pdc-deficient *S. cerevisiae* strains.

Keywords: 2,3-Butanediol, *Saccharomyces cerevisiae*, NADH oxidase, cofactor engineering, pyruvate decarboxylase, fed-batch fermentation

Student ID : 2008-21348

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1.1. Redox metabolism in *Saccharomyces cerevisiae*

Nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are cofactors with different functions in the *S. cerevisiae* metabolic network. These cofactors are preferentially used in dissimilatory (NADH) and assimilatory (NADPH) pathways. This is not always the case, however, several NADH-linked reductions occur in the conversion of central metabolites (pyruvate, oxaloacetate, acetyl-CoA) to cellular monomers such as lipid or amino acid biosynthesis. During growth of *S. cerevisiae* on sugars, the dissimilatory pathways (conversion of acetaldehyde to ethanol and reduction of the quinone pool of respiratory chain) are fully activated (Bakker et al., 2001). In contrast to various other yeasts, *S. cerevisiae* cannot use NADPH as an electron donor to the respiratory chain (Bruinenberg et al., 1985; González et al., 1996; Van Urk et al., 1989). In addition, since the *S. cerevisiae* lacks a transhydrogenase activity which catalyzes the conversion of NAD^+ and NADPH to NADH and $NADP^+$ (Bruinenberg et al., 1985; Camougrand et al., 1988), the pentose phosphate pathway, which produces NADPH primarily cannot function as the dissimilatory route in *S. cerevisiae* (González et al.,

1996). Therefore, the role of NADH is predominantly important in fermentative sugar dissimilation in *S. cerevisiae*.

Under strictly anaerobic conditions, substrate level phosphorylation in glycolysis is the only route for ATP synthesis in *S. cerevisiae* and produces a net yield of two moles of ATP per one mole of glucose converted to two moles of pyruvate. Dissimilation of glucose into pyruvate via the glycolytic pathway is stoichiometrically linked to the reduction of NAD^+ in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction. A redox balance in dissimilation is achieved by the decarboxylation reaction of pyruvate to acetaldehyde by pyruvate decarboxylase. Produced acetaldehyde subsequently acts as the electron acceptor for reoxidation of NADH. In wild type *S. cerevisiae*, ethanol formation is the main fermentative metabolism.

S. cerevisiae exhibits a mixed respiro-fermentative metabolism even in fully aerobic conditions except for a limited sugar supply at low specific growth rates (Kappeli, 1986; Petrik et al., 1983; Postma et al., 1989). This phenomenon of aerobic fermentation is called 'Crabtree effect' which is at least partly because of repression of the synthesis of respiratory enzymes by excess glucose (De Deken, 1966; Petrik et al., 1983; van Dijken & Scheffers, 1986). During respiratory growth on

sugars, the NADH formed by glycolysis can be oxidized via mitochondrial respiration, thus yielding additional ATP via oxidative phosphorylation. Respiratory reoxidation of glycolytic NADH means that pyruvate does not have to be converted into acetaldehyde, but can be further oxidized. Oxidation of pyruvate to acetyl-CoA predominantly occurs via the mitochondrial pyruvate-dehydrogenase (PDH) complex (Pronk et al., 1994). Subsequently, acetyl-CoA is oxidized to carbon dioxide through the tricarboxylic acid (TCA) cycle.

A typical chemical composition of yeast is $C_{3.75}H_{66}N_{0.63}O_{2.10}$ (Verduyn et al., 1990), slightly more reduced than glucose. Therefore, assimilation of carbon sources such as the conversion of glucose into biomass should result in a net input of reducing equivalents. In practice, net consumption of NADPH and net production of NADH are accompanied with biomass formation. The total process yields reducing equivalents in the form of NADH, because assimilation involves production of carbon dioxide (Gommers et al., 1988). The exact magnitude of this assimilatory NADH formation depends on the composition of the biomass and the extent of exogenous lipids (Andreasen & Stier, 1954). In addition to the formation of biomass, the excretion of oxidized metabolites such as pyruvate, acetaldehyde, and

acetate during growth on glucose leads to net production of NADH (Nissen et al., 1997; Oura, 1977; van Dijken & Scheffers, 1986).

S. cerevisiae lacks transhydrogenase activity (Bruinenberg et al., 1985; van Dijken & Scheffers, 1986) and the mitochondrial inner membrane is impermeable to redox pyridine nucleotide molecules such as NADH, NAD^+, NADPH, and $NADP^+$ (Masako & Ishibashi, 1979). Therefore, the reduced form of cofactors should be reoxidized in the compartment where they are produced. NADH turnover occurs at high rates in the cytosol as well as in the mitochondrial matrix (Moore & Jöbsis, 1970; Wilson & Felgner, 1977). During respiratory growth on sugars, NADH is generated not only in the cytosol by glycolysis, but also in the mitochondrial matrix from the TCA cycle. The relative rates of NADH turnover strongly depend on the carbon source. For example, several processes resulted in production of NADH (Blomberg et al., 1988; Fiechter & Seghezzi, 1992; Sprague & Cronan, 1977) contrary to ethanol fermentation which is redox neutral. The largest part of excess cytosolic NADH formation in wild type *S. cerevisiae* is biomass production (Albers et al., 1996; Bruinenberg et al., 1983b). Synthesis of proteins, nucleic acids, and fatty acids were associated with the assimilatory NADH formation.

Anaerobically, *S. cerevisiae* can reoxidize surplus NADH by glycerol production as mentioned previously (Albers et al., 1998; Nordström, 1968; van Dijken & Scheffers, 1986). Aerobically, several systems for conveying excess cytosolic NADH to the mitochondrial electron transport chain exist in *S. cerevisiae*. The two important systems in this respect are the external NADH dehydrogenase (Nde1p/Nde2p) (Luttik et al., 1998; Small & McAlister-Henn, 1998) and the glycerol-3-phosphate shuttle (Larsson et al., 1998). The Nde1 and Nde2 proteins localized in the inner mitochondrial membrane with the catalytic sites projecting towards the intermembrane space of mitochondria directly oxidize cytosolic NADH (Rigoulet et al., 2004). The glycerol-3-phosphate shuttle system involving FAD-dependent Gut2p (Rønnow & Kielland-Brandt, 1993), is also shown to be active in maintaining a cytosolic redox balance (Larsson et al., 1998). In this cofactor system, NADH is oxidized to NAD^+ by the cytosolic glycerol-3-phosphate dehydrogenase (Gpd1p) when catalyzing the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). Subsequently G3P conveys electrons to the respiratory chain via Gut2p and is converted again into DHAP. Even if the two systems are available, the capacity of the Gut2p is not enough to completely replace

Nde1p/Nde2p activity when NADH flux is high. On the other hand, Gut2p shuttle is more efficient in producing ATP. Therefore, the activities of intermembrane dehydrogenases might be under concerted regulation (Rigoulet et al., 2004).

1.2. Cofactor engineering strategies in *S. cerevisiae*

S. cerevisiae is the most thoroughly investigated eukaryotic microorganism. Especially, this organism has been used in a number of different processes within the chemical and pharmaceutical industries (Ostergaard et al., 2000). *S. cerevisiae* is a very attractive organism because it is nonpathogenic, and due to its long history of applications in the production of ethanol, it has been classified as a generally recognized as safe (GRAS) organism. Also, the well-established fermentation and process technology for large-scale fermentation with *S. cerevisiae* make this organism attractive for several biotechnological uses. In addition, various genetic modifications are applicable because of the availability of the complete genome sequence and genetic tools, and information about metabolic systems. Cofactor engineering is necessary for efficient production of chemicals using *S. cerevisiae* as a host strain. As mentioned above, *S. cerevisiae* does not have any

transhydrogenase activity (Bruinenberg et al., 1985; Bruinenberg et al., 1983a), and the mitochondrial inner membrane is impermeable to redox cofactors such as NADH and NADPH (Jagow & Klingenberg, 1970). In addition, relieving the compartmentation of cofactors between mitochondria and cytosol is not sufficient because of the tight regulated cofactor shuttle and electron transport chain by aeration conditions and glucose-sensing mechanism (Bakker et al., 2001; Gancedo, 1998; van Dijken & Scheffers, 1986). Therefore, the cofactor engineering strategies using transhydrogenase, NADH kinase, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH), NADH oxidase, and modulation of the innate metabolic pathways have been processed for the efficient production of various chemical compounds.

Pyridine dinucleotide transhydrogenases catalyze the direct and reversible transfer of a hydrogen ion equivalent between oxidized and reduced forms of NAD and NADP according to the equation : NADH + $NADP^+$ → NAD^+ + NADPH (Everse, 2012). Two types of transhydrogenases have been identified (Rydstrom et al., 1976). AB-specific transhydrogenases, acting on NADH or NADPH with NAD^+ or $NADP^+$ as acceptor, are membrane-bound and catalyze the reaction under simultaneous translocation of protons across a membrane (Hoek

& Rydstrom, 1988). BB-specific transhydrogenases acting non-energy-linked reaction occurred in the cytoplasm and are independent of proton translocation (Anderlund et al., 1999). They have been found in the bacteria *E. coli*, *P. fluorescens*, *P. aeruginosa*, and *A. vinelandii* (Boonstra et al., 1999; French et al., 1997). There were several attempts for cofactor engineering of *S. cerevisiae* through the expression of transhydrogenases. The expression of soluble transhydrogenase (STH) of *A. vinelandii* reduced xylitol productivity because the reaction directed toward the formation of NADH and $NADP^+$ from NAD^+ and NADPH, which concomitantly reduced the availability of NADPH for xylose conversion to xylitol catalyzed by NADPH-preferable xylose reductase in the recombinant *S. cerevisiae* (Jeun et al., 2003). When plasma membrane-bound transhydrogenase of *E. coli* was transformed into *S. cerevisiae*, a functional protein was synthesized that presumably accumulated in the rough endoplasmic reticulum (ER). Unfortunately, this transhydrogenase favored NADH and $NADP^+$ formation, and hence an increased glycerol yield and a decreased ethanol yield were observed (Anderlund et al., 1999). Expression of cytoplasmic transhydrogenase from *A. vinelandii* in *S. cerevisiae* resulted in the changed intracellular redox state and product formation.

(NADPH/$NADP^+$):(NADH/NAD^+) ratio was reduced from 35 to 17 by expression of transhydrogenase and resulted in significantly reduced biomass and ethanol yield (Nissen et al., 2001). In addition, the research with a double *gpd1 gpd2* deletion mutant strain to introduce a new pathway to regenerate NAD^+ was attempted by expressing bacterial transhydrogenase of *A. vinelandii*. Unfortunately, the NAD^+ pool became limiting for biomass synthesis before the transhydrogenase was able to support the synthesis of NAD^+, and consequently no growth was observed under anaerobic conditions (Nissen et al., 2000a). The applications of transhydrogenase in cofactor engineering seemed to be limited because the direction of transhydrogenase activity cannot be controlled *in vivo*.

The NADH kinases convert NAD(H) to NADP(H) by the ATP-linked phosphorylation (McGuinness & Butler, 1985). Most multicellular eukaryotic organisms have only one NAD(H) kinase gene (Shianna et al., 2006). All of these NAD(H) kinases are highly conserved from *E. coli* to mammals (Lerner et al., 2001). In contrast, *S. cerevisiae* has

three NAD/H kinase genes (*UTR1*, *YEF1*, *POS5*). All of these genes have been shown biochemically to encode functional NAD(H) kinases.

Utr1p localizes to the cytoplasm and preferentially converts NAD to NADP (Kawai et al., 2001). Yel041w (Yef1p) has recently been shown to be ATP–NADH kinase (Shi et al., 2005). Pos5p localizes to the mitochondria (Outten & Culotta, 2003) and preferentially converts NADH to NADPH (Kawai et al., 2001; Strand et al., 2003). Overexpression of NADH kinase encoded by the *POS5* gene in the mitochondria was ineffective for the anaerobic growth of xylose-utilizing *S. cerevisiae*, and in the cytosol, it increases xylitol accumulation (Hou et al., 2009b). However, combined expression of the heterologous *gapN* pathway from *S. mutans*, deletion of the *FPS1* gene (glycerol export) and overexpression of NADH kinase (Utr1) reduced glycerol formation and increased ethanol yield (Navarrete et al., 2014). In addition, by co-expression of glucose-6-phosphate dehydrogenase (Zwf1) and NADH kinase (Pos5) into a carotenoid-producing *S. cerevisiae* strain, the yield of lycopene and β-carotene increased substantially (Zhao et al., 2015). $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase (NADP-GAPDH) has been used for cofactor engineering in *S. cerevisiae*. Since the endogenous GAPDH enzyme catalyzes glyceraldehyde-3-phosphate with NADH as a cofactor, the expression of $NADP^+$-dependent GAPDH converted

cytosolic NADH to NADPH as transhydrogenase. The overexpression of the *K. lactis GDP1* gene (Verho et al., 2002) encoding an NADP-GAPDH in a xylose-fermenting *S. cerevisiae* strain enhances ethanol production (Verho et al., 2003). Similarly, nonphosphorylating $NADP^+$-dependent GAPDH, encoded by the *gapN* gene, from *S. mutants* in xylose metabolizing *S. cerevisiae* increased ethanol production by using a genome-scale metabolic flux model (Bro et al., 2006).

Water-forming NADH oxidase 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 (de Felipe et al., 1998a; Geueke et al., 2003; Hummel & Riebel, 2003; Kawasaki et al., 2004; Matsumoto et al., 1996; Riebel et al., 2003; Schmidt et al., 1986). Among them, *noxE* from *L. lactis* (Heux et al., 2006; Hou et al., 2014; Kim et al., 2015; Zhang et al., 2012a) and *nox* from *S. pneumoniae* (Hou et al., 2009a; Su et al., 2015; 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 (Hou et al., 2009a; Kim et al., 2015; 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 addition, overexpression of *noxE* in xylose-fermenting *S. cerevisiae* significantly decreased xylitol and glycerol formation and increased final ethanol concentration during xylose fermentation (Zhang et al., 2012a). Similarly, by fine-tuning NADH oxidase expression, the glycerol or/and xylitol production were reduced in both recombinant *XI* and *XR-XDH* xylose-metabolizing yeast strains (Hou et al., 2014). Maintaining redox balance is also crucial for the production of heterologous secondary metabolites. The redox imbalance could be alleviated by water-forming NADH oxidase and alternative oxidase and the production of 7-dehydrocholesterol substantially increased (Su et al., 2015).

Modulation of innate metabolic pathways can be applied to cofactor engineering for enhanced chemical production. These pathways include ammonium assimilation and malate, formate, and fumarate production. Redirection of flux toward ethanol has been achieved by modifying the ammonium assimilation pathway (Kim et al., 2012; Kong et al., 2006; Kong et al., 2007; Nissen et al., 2000c). Higher ethanol production

accompanying reduced glycerol production can also be obtained by deleting the NADPH-dependent glutamate dehydrogenase gene *GDH1* and overexpressing the NADH-dependent isozyme gene *GDH2*. These strategies to redirect flux towards ethanol formation appear to improve intracellular cofactor concentrations in *S. cerevisiae* (Nissen et al., 2000c). In addition, overexpression of the GS-GOGAT complex including *GDH1*, *GDH2*, *GLT1* and *GLN1* increased ethanol yield in carbon-limited continuous cultivation at a low dilution rate in xylose fermentation (Roca et al., 2003). Reducing glycerol production by modulation of the ammonium assimilation pathway has a synergetic effect with the deletion of the glycerol biosynthetic genes (Kim et al., 2012) or glycerol exporter (Kong et al., 2006). A transhydrogenase-like cofactor shunt composed of malic enzyme (*MAE1*), malate dehydrogenase (*MDH2*), and pyruvate carboxylase (*PYC2*) was overexpressed in xylose-fermenting *S. cerevisiae* (Moreira dos Santos et al., 2004; Suga et al., 2013). The redox state of the resulting strains was changed dramatically, but resulted in poor ethanol yield as well as increased production of xylitol (Suga et al., 2013). The overexpression of the *FDH1* gene encoding formate dehydrogenases and the *GPD2* gene encoding glycerol-3-phosphate dehydrogenase increased

intracellular NADH/NAD^+ ratio (Geertman et al., 2006). In addition, expression of fumarate reductase could provide an important source of NAD^+ under anaerobic condition for relieving redox imbalance by *S. cerevisiae*. The expression of glycosomal NADH-dependent fumarate reductase (*FRD*) of *T. brucei* in xylose-utilizing *S. cerevisiae* together with cytosolic NADH kinase (*POS5Δ17*) substantially increased ethanol yield (Salusjärvi et al., 2013).

1.3. 2,3-Butanediol

2,3-Butanediol (2,3-BD) is a chemical compound that has a molecular weight of 90.12 kDa. 2,3-BD is also called as 2,3-butylene glycol, 2,3-dihydroxybutane, dimethylethylene glycol, or butan-2,3-diol (Syu, 2001). 2,3-BD is a multi-functional platform chemical that has broad industrial applications. 2,3-BD could be used as an antifreeze agent (levo- form of 2,3-BD) because of its low freezing point (-60^{o}C), an “octane booster” for petroleum fuels due to its high octane number (Celińska & Grajek, 2009), a moisturizer (Simion et al., 2005), and a plant growth hormone (Ryu et al., 2003). Moreover, derivatives of 2,3-BD by dehydrogenation, ketalization, esterification, and dehydration could be applied for production of various commercial products (Syu,

2001). 1,3-Butadiene which could be prepared from 2,3-BD by dehydration is a substance used for the production of synthetic rubber. Methyl ethyl ketone, another dehydration product of 2,3-BD, is regarded as an effective liquid fuel additive and used as a solvent for resins and lacquers (Flickinger, 1980; Haveren et al., 2008; Syu, 2001). Polyurethane-maleamides (PUMAs) synthesized by esterification of 2,3-BD can make cardiovascular medical devices (Petrini et al., 1999). Additionally, esterified 2,3-BD can be used of precursors for drugs, cosmetics, and plasticizers (Garg & Jain, 1995). Diacetyl and acetoin produced by dehydrogenation of 2,3-BD could be used as flavoring agents and food additives (Bartowsky & Henschke, 2004). Above this, 2,3-BD and derivatives form of 2,3-BD have potential applications such as moistening and softening agents, printing inks, perfumes, spandex, and carrier for pharmaceuticals (Garg & Jain, 1995).

The global market for 2,3 butanediol was estimated to be 58 kilo tons in 2010, which is expected to reach market volumes of 74.4 kilo tons by 2018, growing at a compound annual growth rate (CAGR) of 3.2% from 2013 to 2018 (http://www.transparencymarketresearch.com). In addition, because of concerns about the impact of using petroleum-based 2,3-butanediol on environment, the market is shifting towards the

use of bio-based 2,3-butanediol providing a clean solution for producing various derivatives of chemicals.

Biological production of 2,3-BD has a history more than a century. Through the fermentations with microorganisms such as *K. pneumoniae* (Donker, 1926) and *B. polymyxa* (Harden & Walpole, 1906), microbial production of 2,3-BD has been started. Owing to a need for synthetic rubber during World War II, production of 2,3-BD and subsequently 1,3-butadiene became more important than ever. However, as petroleum-based routes became available, further improvements and production were ceased. In the 1970s, noteworthy interests in microbial production of 2,3-BD from biomasses were revived by the long-term prospects of rising petroleum prices (Voloch M, 1985). Because of unstable fossil fuel supplies and prices and environmental issues, microbial production of 2,3-BD was paid great attention nowadays (Hatti-Kaul et al., 2007; John et al., 2007; Kamm & Kamm, 2004; Zverlov et al., 2006).

2,3-BD can exist in three isomeric forms: (2S, 3S)-, meso-, and (2R, 3R)- forms (Figure 1-1). Because it is complicated and expensive to select specific stereoisomers in chemical processes, production of 2,3-BD via biological routes is preferable to obtain pure stereoisomers of

2,3-BD (Liu et al., 2011; Ui et al., 2004). Each 2,3-BD-producing microorganisms has a connatural spectrum of stereoisomers as described in Table 1-1 and Table 1-2. Generally, a mixture of two stereoisomers is formed (Maddox, 1996). For example, *K. pneumoniae*, *K. oxytoca*, and *E. aerogenes* produce a mixture of (2S, 3S)-BD and meso-2,3-BD (Afschar et al., 1991; Ma et al., 2009; Zeng et al., 1991). *B. licheniformis* and *B. amyloliquefaciens* produce a mixture of (2R, 3R)-BD and meso-2,3-BD (Gao et al., 2010; Nilegaonkar et al., 1992). Especially, *P. polymyxa* produces pure form of (2R, 3R)-BD (Häßler et al., 2012) and *S. marcescens* produces pure form of meso-2,3-BD (Zhang et al., 2010). As the mechanisms about the formation of stereoisomers were not fully revealed, it is regarded as the result of the specificity of 2,3-butanediol dehydrogenases (Chen et al., 2014; González et al., 2010; Ji et al., 2011).

1.4. Production of 2,3-BD by native producers

As shown in Table 1-1, several bacterial strains were reported as a native 2,3-BD producer (Garg & Jain, 1995). *K. pneumoniae*, *K. oxytoca*, and *E. aerogenes* produced 2,3-BD with high titer, yield and productivity (Ma et al., 2009; Qureshi & Cheryan, 1989; Zeng et al.,

1994). In addition, *B. amyloliquefaciens* and *P. polymyxa* are also regarded as promising 2,3-BD production strains (Gao et al., 2010; Yang et al., 2011).

Bacterial strains produce 2,3-BD via a mixed acid fermentation. Along with 2,3-BD, organic acids such as formate, lactate, succinate, and acetate are produced and ethanol is also produced as a byproduct (Maddox, 1996; Magee & Kosaric, 1987). As shown in Figure 1-2, pyruvate formed by the glycolysis of sugars is converted into α-acetolactate by α-acetolactate synthase (*ALS*). Then, acetoin is synthesized from α-acetolactate via α-acetolactate decarboxylase (*ALDC*). Finally, acetoin is converted into 2,3-BD by butanediol dehydrogenase (*BDH*) which is also known as acetoin reductase (Celińska & Grajek, 2009).

A large number of native producers for 2,3-BD are classified as risk group II (pathogenic). Although *P. polymyxa* is a nonpathogenic native 2,3-BD producer, lack of genetic tools and difficulty in medium optimization for preventing biofilm hinder the development of a 2,3-BD production host strain (Kim & Timmusk, 2013; Li et al., 2013). In addition, bacterial strains are at a risk of bacteriophage infection in large-scale fermentations for industrial production (Wêgrzyn, 2004).

1.5. Production of 2,3-BD by engineered strains

Recently, a number of studies were carried out to overcome limitations of bacterial 2,3-BD native producers such as various byproduct formation, strain safety, and lack of stereospecificity (Table 2-2). *E. cloacae* was metabolically engineered to produce 2,3-BD efficiently from lignocellulosic hydrolysates through inactivation of the glucose transporter encoding the gene for *ptsG* and overexpression of the galactose permease encoding gene *galP* (Li et al., 2015). Metabolic engineering was applied to *K. oxytoca* to improve 2,3-BD yield by reducing byproducts formation. Deletion of alcohol dehydrogenase E (*adhE*), acetate kinase A-phosphotransacetylase (*ackA-pta*), and lactate dehydrogenase A (*ldhA*) increased 2,3-BD yield with only trace amounts of acetoin, lactate, formate, acetate and ethanol (Jantama et al., 2015). In addition, deletion of the *aldA* gene coding for aldehyde dehydrogenase in *K. oxytoca* led to significantly improved 2,3-BD production (Ji et al., 2010). *E. coli* which is not a native producer of 2,3-BD was engineered to produce 2,3-butanediol by introduction of the 2,3-BD biosynthetic gene clusters from native producers (Li et al., 2010; Xu et al., 2014). Gene knock-out of the *budC* gene in *B.*

licheniformis blocked meso-2,3-BD production and produced pure isomer of (2R, 3R)-BD (Qi et al., 2014). *B. amyloliquefaciens* which is a GRAS strain was isolated to produce 2,3-BD (Yang et al., 2011). UV or chemical mutagenesis of *S. marcescens* and *K. oxytoca* reduced organic acid formation and increased 2,3-BD titer and yield (Han et al., 2013; Zhang et al., 2010). Overexpression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and 2,3-butanediol dehydrogenase (*BDH*) in *B. amyloliquefaciens* increased 2,3-BD yield and concentration (Yang et al., 2013). Recently, in addition to genetic engineering of bacterial strains, baker's yeast *S. cerevisiae* was engineered to produce 2,3-BD. Based on *in silico* genome-scale metabolic analysis, genes of alcohol dehydrogenase (*ADH1, ADH3 and ADH5*) were deleted and the 2,3-BD biosynthetic genes from *B. subtilis* and *E. aerogenes* were introduced. The engineered *S. cerevisiae* produced 2.29 g/L of 2,3-BD at 0.113 g/g of yield (Ng et al., 2012).

1.6. 2,3-BD biosynthesis in pyruvate decarboxylase-deficient *S. cerevisiae*

Although wild type *S. cerevisiae* strains could produce 2,3-BD naturally, concentration, yield and productivity of 2,3-BD were

extremely low compared to native bacterial producers. Thus, *S. cerevisiae* was not regarded as a 2,3-BD producing strain (Garg & Jain, 1995). 2,3-BD is synthesized by *S. cerevisiae* from the isoleucine and valine synthetic pathway in mitochondria. Pyruvate produced by the glycolysis of carbon sources is transported to mitochondria and converted into α-acetolactate by acetolactate synthase (Ilv2). α-acetolactate is not converted into acetoin enzymatically different from bacterial strains since α-acetolactate decarboxylase does not exist in *S. cerevisiae*. Alternately, α-acetolactate reacts with molecular oxygen and converts into diacetyl called the spontaneous decarboxylase reaction (Figure 1-3). Then, acetoin was formed by a reaction of diacetyl reductase which encoded as *BDH1* in *S. cerevisiae*. Finally, Bdh1p which is 2,3-butanediol dehydrogenase converts acetoin to 2,3-BD. Generally, 2,3-BD accumulates during wine fermentation at concentrations ranging from 0.2 to 3.0 g/L (Ehsani et al., 2009; Sponholz et al., 1993). Studies on the 2,3-BD production pathway in *S. cerevisiae* have been progressed for enhancing flavors in beer or wine fermentations (Romano & Suzzi, 1996; Sponholz et al., 1993). The introduction of α-acetolactate decarboxylase produced only a trace amount of 2,3-BD (Suihko et al., 1990; Vanderhaegen et al., 2003).

Recently, high titer of 2,3-BD was produced through the engineered pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* (Kim et al., 2013a). *S. cerevisiae* predominantly produces ethanol from sugar fermentation especially from glucose. To maximize the production of lactic acid, glycerol and malic acid by *S. cerevisiae*, Pdc-deficient *S. cerevisiae* has been employed in the other studies (Geertman et al., 2006; Ishida et al., 2006; Zelle et al., 2008). Pyruvate which is produced by the glycolysis of sugars is located at the branch point of the fermentative and respiratory metabolisms. In the fermentative metabolism, pyruvate is converted into acetaldehyde by Pdc and further reduced into ethanol by alcohol dehydrogenase (Adh). Pyruvate decarboxylase was encoded by three structural genes (*PDC1*, *PDC5*, and *PDC6*). The deletion of these genes led to loss of pyruvate decarboxylase activity and could not produce ethanol from fermentable sugars (Pronk et al., 1996).

Pdc-deficient *S. cerevisiae* strains have a growth defect by two reasons mainly. First, the Pdc-deficient *S. cerevisiae* requires supplementation of C_2-compounds such as ethanol, acetate, and acetaldehyde for synthesis of cytosolic acetyl-CoA which is required to synthesize lysine and fatty acids (Flikweert et al., 1999; Flikweert et al.,

1996; Pronk et al., 1996). Cytosolic acetyl-CoA is synthesized from acetate by acetyl-CoA synthetase (Acs) in *S. cerevisiae*. Second, the Pdc-deficient *S. cerevisiae* showed lower growth rate in glucose medium than wild type *S. cerevisiae* because of cofactor imbalance in cytosol. One of the role of ethanol production by *S. cerevisiae* is regeneration of NAD^+ from NADH which is formed by glycolysis. Since a rate of NADH oxidation by the respiratory metabolism in *S. cerevisiae* is not fast enough to relative for the fermentative metabolism, elimination of ethanol formation of Pdc-deficient *S. cerevisiae* accumulated NADH and thereby cofactor imbalance in cytosol.

Pdc-deficient *S. cerevisiae* strains which could grow on glucose medium were reported recently (Kim et al., 2013a; Oud et al., 2012; van Maris et al., 2004). These strains were constructed by evolutionary engineering of Pdc-deficient *S. cerevisiae* in glucose medium. The resulting strains have the mutant *MTH1* gene which is involved in glucose sensing signaling pathway. Through introduction of the 2,3-BD biosynthetic pathway into the evolved Pdc-deficient *S. cerevisiae* strain, high titer of 2,3-BD (96.2 g/L) was produced. However, the 2,3-BD yield and productivity were still substantially lower than the native

bacterial strains for 2,3-BD production because of slow glucose uptake rates and a large amount of glycerol formation as a byproduct.

1.7. Objectives of the dissertation

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

1) Production of 2,3-BD without ethanol formation through the introduction of the 2,3-BD biosynthetic enzymes such as α-acetolactate synthase (*alsS*), α-acetolactate decarboxylase (*alsD*) and 2,3-butanediol dehydrogenase (*BDH1*) into Pdc-deficient *S. cerevisiae*.

2) Improvement of 2,3-BD titer, yield and productivity by i) modulation of intracellular cofactor levels through expression of NADH oxidase and control of aeration and ii) fine-tuning of pyruvate decarboxylase activity of Pdc-deficient 2,3-BD-producing *S. cerevisiae* strains.

3) Elimination of glycerol formation as a by-product through the deletion of glycerol-3-phosphate dehydrogenase genes (*GPD1* and *GPD2*).

4) Optimization of a fermentation process for efficient production of 2,3-BD by engineered *S. cerevisiae*.

Table 1-1. 2,3-BD production with microbial strains

Strains	Description	Stereo isomer	Carbon source	Methods	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	Reference
Klebsiella pneumoniae CICC 10011	Simultaneous saccharification and fermentation (SSF)	(2S, 3S)-, meso-	Glucose	Fed-batch	91.6	2.29	0.32*	(Sun et al., 2009)
Klebsiella pneumoniae SDM	Medium optimization	(2S, 3S)-, meso-	Glucose	Fed-batch	150	4.21	0.47	(Ma et al., 2009)
Klebsiella pneumoniae G31	(2S, 3S)-, meso-	(2S, 3S)-, meso-	Glycerol	Fed-batch	45.8	0.18	0.42	(Petrov & Petrova, 2009)
Klebsiella pneumoniae SDM	Utilization of corncob molasses	(2S, 3S)-, meso-	Glucose, xylose and arabinose	Fed-batch	82.5	1.35	0.42	(Wang et al., 2010)
Klebsiella oxytoca NRRL B-199	Culture optimization	(2S, 3S)-, meso-	Glucose	Fed-batch	85.5	2.89	0.45	(Qureshi & Cheryan, 1989)
Klebsiella oxytoca NRCC 3006	Utilization of molasses	(2S, 3S)-, meso-	Glucose, fructose	Batch	95.2	1.0	0.47	(Afschar et al., 1991)

Klebsiella oxytoca ATCC 8724	Simultaneous saccharification and fermentation (SSF)	(2S, 3S)-, meso-	Cellulose, xylan	Batch	25.0	0.36	0.31	(Cao et al., 1997)
Klebsiella oxytoca ATCC 10370	Medium optimization	(2S, 3S)-, meso-	Xylose, glucose, arabinose, cellobiose	Fed-batch	35.7	0.59	0.50	(Cheng et al., 2010)
Enterobacter aerogenes DSM 30053	Cell recycle system	(2S, 3S)-, meso-	Glucose	Fed-batch	110	5.4	0.48	(Zeng et al., 1991)
Bacillus licheniformis	Culture optimization	(2R, 3R)-, meso	Glucose	Batch	8.7	0.12	0.47	(Nilegaonkar et al., 1992)
Paenibacillus polymyxa ZJ-9	Medium optimization	(2R, 3R)-	Inulin	Batch	36.9	0.88	0.50	(Gao et al., 2010)
Bacillus amyloliquefaciens B10-127	Strain selection	(2R, 3R)-, meso	Glucose	Fed-batch	92.3	0.96	0.42	(Yang et al., 2011)
Paenibacillus polymyxa DSM 365	Medium optimization	(2R, 3R)-	Sucrose	Fed-batch	111	2.05	-	(Häßler et al., 2012)

* Yield of 2,3-butanediol + acetoin

Table 1-2. 2,3-BD production with engineered strains

Strains	Description	Stereo isomer	Carbon source	Methods	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	Reference
Enterobacter cloacae SDM	*ptsGΔ ldhΔ frdAΔ galP* overexpression	(2R,3R)-	Glucose, xylose	Fed-batch	152.0	3.5	0.49	(Li et al., 2015)
			Lignocellulosic hydrolysates	Fed-batch	119.4	2.3	0.47	(Li et al., 2015)
Klebsiella oxytoca KMS005	*adhEΔ ackA-ptaΔ ldhAΔ*	(2S,3S)-, meso	Glucose	Fed-batch	117.4	1.20	0.49	(Jantama et al., 2015)
Escherichia coli BL21(DE3)	2,3-BD biosynthetic gene clusters from native producers	Meso-	Glucose	Fed-batch	73.8	1.19	0.45	(Xu et al., 2014)
Escherichia coli JM109	*ldhAΔ ptaΔ adhEΔ poxBΔ alsS alsD* from *B. subtilis budC* from *K. pneumoniae*	Meso-	Glucose	Batch	9.5	0.20	0.33	(Li et al., 2010)
Bacillus licheniformis WX-02	*budCΔ*	(2R,3R)-	Glucose	Batch	30.8	1.28	0.26	(Qi et al., 2014)

Saccharomyces cerevisiae D452-2	*pdc1Δ pdc5Δ gh1-1 cdt-1* from *Neurospora crassa alsS*, *alsD* from *Bacillus subtilis*	(2R,3R)-	Cellobiose	Batch	5.29	0.22	0.29	(Nan et al., 2014)
Saccharomyces cerevisiae D452-2	*pdc1Δ pdc5Δ alsS*, *alsD* from *Bacillus subtilis XYL1*, *XYL2*, *XYL3* from *Scheffersomyces stipitis*	(2R,3R)-	Xylose	Batch	20.7	0.18	0.27	(Kim et al., 2014)
Saccharomyces cerevisiae D452-2	*pdc1Δ pdc5Δ alsS*, *alsD* from *Bacillus subtilis*	(2R,3R)-	Glucose	Fed-batch	96.2	0.39	0.28	(Kim et al., 2013a)
Klebsiella oxytoca NBRF4	Chemical mutagenesis	(2S,3S)-, meso-	Glucose	Fed-batch	34.2	0.49	0.35	(Han et al., 2013)
Saccharomyces cerevisiae BY4742	*adh1Δ adh3Δ adh5Δ alsS* from *Bacillus subtilis budA*, *budC* from *Enterobacter aerogenes*	(2R,3R)-, meso-	Glucose	Batch	2.3	0.11	0.03	(Ng et al., 2012)

Klebsiella oxytoca ME-UD-3	*aldAΔ*	(2S,3S)-, meso-	Glucose	Fed-batch	130	1.63	0.48	(Ji et al., 2010)
Serratia marcescens H30	UV and chemical mutagenesis	Meso-	Glucose	Fed-batch	139.9	3.49	0.47	(Zhang et al., 2010)
Bacillus subtilis RB03	*budA* expression	(2R,3R)-, meso-	Glucose	Batch	6.1	0.4	0.43	(Biswas et al., 2012)
Bacillus amyloliquefaciens B10-127	*GAPDH BDH* expression	(2R,3R)-, meso-	Glucose	Fed-batch	132.9	2.95	0.42	(Yang et al., 2013)
Saccharomyces cerevisiae CEN PK2-1C	*adh1-5Δ gpd1Δ gpd2Δ* *alsS* and *alsD* from *B. subtilis* *noxE* from *L. lactis*	(2R,3R)-	Glucose	Fed-batch	72.9	1.43	0.41	(Kim & Hahn, 2015)
Saccharomyces cerevisiae CEN PK2-1C	*pdc1Δ pdc5Δ pdc6Δ* *MTH1* expression *alsS* and *alsD* from *B. subtilis* endogenous *BDH1*	(2R,3R)-	Glucose, galactose	Fed-batch	100	0.31	0.35	(Lian et al., 2014)

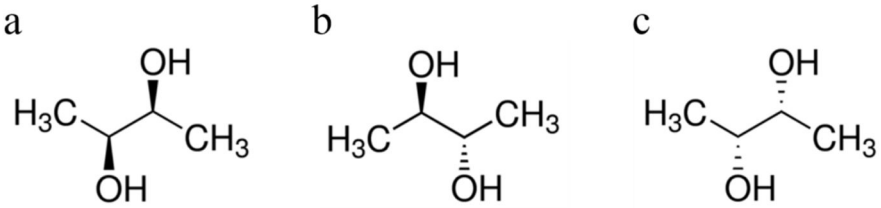


Figure 1-1. Stereoisomers of 2,3-butanediol. (a) (2S,3S)-2,3-butanediol, Dextrorotatory form; (b) meso-2,3-butanediol, Optically inactive form; (c) (2R,3R)-2,3-butanediol, Levorotatory form.

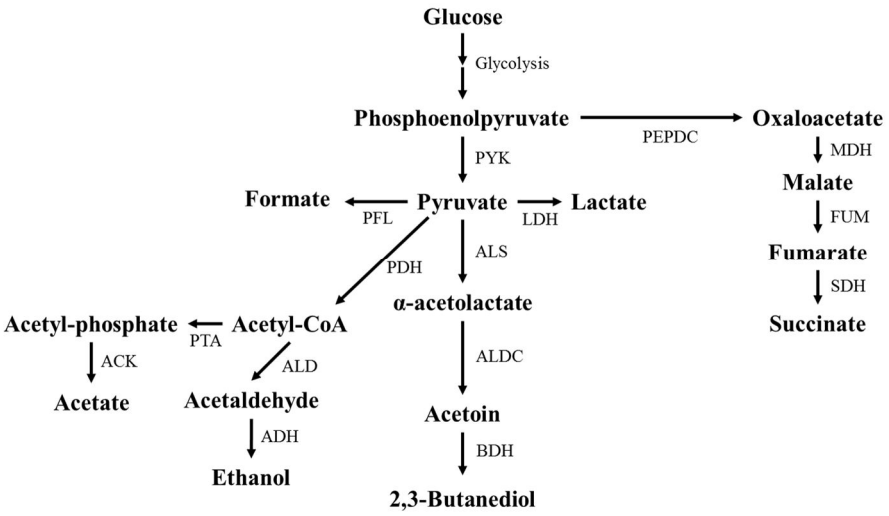


Figure 1-2. Mixed acid-2,3-butanediol pathway in bacteria (modified based on (Ji et al., 2011)). PEPDC, phosphoenolpyruvate decarboxylase; MDH, malate dehydrogenase; FUM, fumarase; SDH, succinate dehydrogenase; PYK, pyruvate kinase; PFL, pyruvate-formate lyase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase complex; ALD, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; PTA, phospho-transacetylase; ACK, acetate kinase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase; BDH, butanediol dehydrogenase.

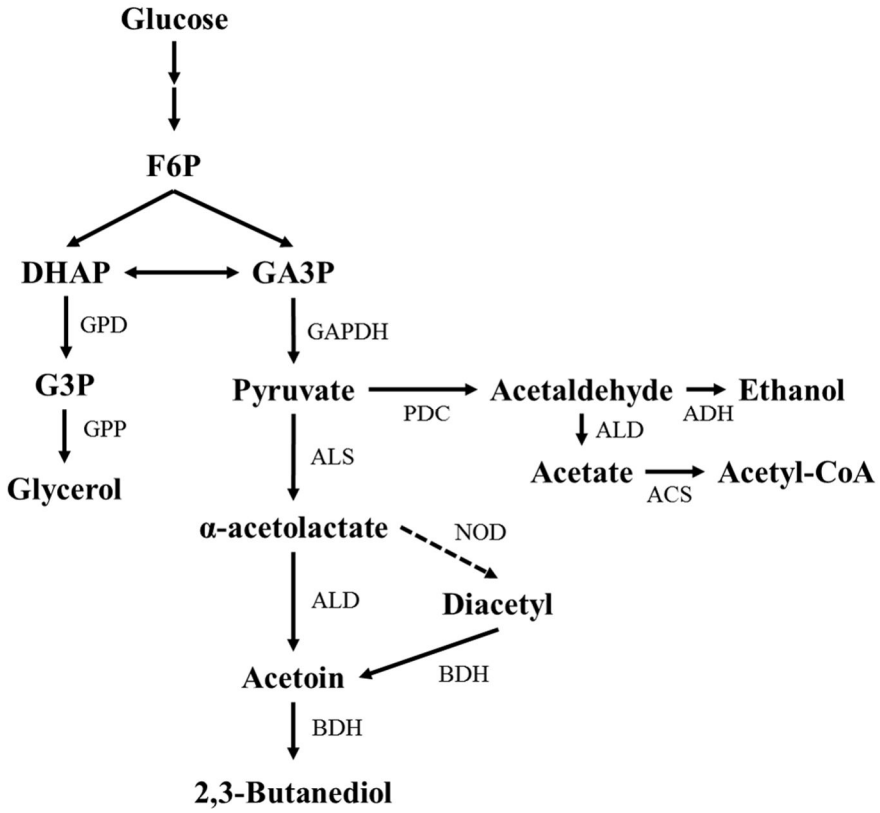


Figure 1-3. 2,3-BD production pathway in *Saccharomyces cerevisiae*. F6P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate; GPD, glycerol 3-phosphate dehydrogenase; GPP, glycerol 3-phosphate phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PDC, pyruvate decarboxylase; ALD, aldehyde dehydrogenase; ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase; NOD, nonenzymatic oxidative decarboxylation; BDH, butanediol dehydrogenase.

Chapter 2.

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

2.1. Summary

2,3-Butanediol (2,3-BD) is a chemical compound with multiple applications in chemical industries. To increase 2,3-BD production, pyruvate decarboxylase genes (*PDC1*, *PDC5*, and *PDC6*) were deleted and the hetelogous 2,3-BD biosynthetic pathway enzymes of α-acetolactate synthase (*alsS*) and α-acetolactate decarboxylase (*alsD*) from *B. subtilis* and endogenous 2,3-butanediol dehydrogenase (*BDH1*) were introduced to the two Pdc-deficient *S. cerevisiae* strains. The 2,3-BD fermentation properties of the resulting strains (BD4 and BD5) were different according to supplementation of ethanol as a C_2-compound because of the modified glucose sensing pathway by *MTH1* mutation. When glucose is used as a sole carbon source, the volumetric productivity of 2,3-BD by the BD4 strain was 1.8-folds higher than that of the BD5 strain. On the other hand, in the presence of ethanol, the volumetric productivity of 2,3-BD by the BD5 strain (0.40 g/L/h) was substantially higher than that of the BD4 strain (0.21 g/L/h). Pdc-deficient *S. cerevisiae* strains could grow on glucose by mutation on the *MTH1* gene because of relieved cofactor imbalance through reduced expression levels of the hexose transporter genes. However, when 2,3-BD biosynthetic enzymes were expressed, the Pdc-deficient *S. cerevisiae* strain (BD5) could grow on glucose despite of absence of mutation on the *MTH1* gene. because cofactor imbalance of the Pdc-deficient *S. cerevisiae* strain

could be relieved by the 2,3-BD biosynthetic pathway. The introduction of the 2,3-BD biosynthetic enzymes and a supplementation of C_2-compound were necessary for Pdc-deficient *S. cerevisiae* to grow on glucose as a sole carbon source.

2.2. Introduction

Native 2,3-BD producers such as *K. pneumoniae*, *K. oxytoca*, *P. polymyxa*, *E. aerogenes* which can produce 2,3-BD with high titer, yield and productivity are regarded as a promising microorganism for industrial uses (Perego et al., 2000). However, most of these bacterial strains are classified as risk group II (pathogenic) microorganisms. In addition, bacterial strains are exposed to risk of biofouling (Mattila-Sandholm & Wirtanen, 1992) and bacteriophage contamination (Marks & Sharp, 2000). Therefore, a reliable process of industrial-scale fermentations with the bacterial strains might be difficult in terms of safety and industrialization. On the other hand, a baker's yeast *S. cerevisiae* is considered as a generally recognized as safe (GRAS) microorganism, and free from the biofilm formation and bacteriophage contamination(Marks & Sharp, 2000; Mattila-Sandholm & Wirtanen, 1992). Thus, the production of 2,3-BD by *S. cerevisiae* could be attractive. Although *S. cerevisiae* has the innate 2,3-BD production pathway, the metabolic fluxes are not high enough for efficient production of 2,3-BD. First, pyruvate produced by the glycolysis pathway is converted into acetaldehyde by high affinity pyruvate decarboxylases in *S. cerevisiae*, and hence is converted to ethanol predominantly. Second, Ilv2, α-acetolactate synthase of *S. cerevisiae*, is located in mitochondria and has a low affinity for α-acetolactate (Falco et al., 1985). Third, *S. cerevisiae* cannot convert α-

acetolactate into acetoin efficiently because acetoin is formed by spontaneous decarboxylation of α-acetolactate unlike the enzymatic reaction of bacterial strains. Thus, for efficient production of 2,3-BD by *S. cerevisiae*, introduction of exogenous 2,3-BD biosynthetic pathway is necessary.

Pdc-deficient *S. cerevisiae* is an attractive 2,3-BD production host strain because ethanol formation is blocked and a large flux of pyruvate can be supplied for 2,3-BD synthesis. However, absence of Pdc activity led to a growth defect by lack of C_2-compounds for acetyl-CoA synthesis and cofactor imbalance by accumulation of cytosolic NADH (Flikweert et al., 1996). In the previous studies, mutation on the *MTH1* gene which encodes a negative regulator of the glucose signaling pathway is used for a promising strategy to overcome growth defect of the Pdc-deficient *S. cerevisiae* on glucose (Kim et al., 2013a; Oud et al., 2012; van Maris et al., 2004). Internal deletion in the *MTH1* gene was identified in the TAM strain by which a phosphorylation site and putative PEST regions were missed (Oud et al., 2012). Also, the evolved Pdc-deficient strain which could grow on high concentrations of glucose without supplementation of C_2-compounds was also constructed (SOS4). Genome sequencing of the SOS4 strain revealed a point mutation (G241C) on the *MTH1* gene (Kim et al., 2013a). Mth1 represses the transcription of the *HXT* genes together with a transcriptional regulator of Rgt1 when extracellular glucose is absent. With extracellular

excess glucose, Mth1 is phosphorylated by casein kinase I (Yck1/2) activated by the signal from the Rgt2/Snf3 glucose sensors, thereby Mth1 is degraded. Degradation of Mth1 allows de-repression of the *HXT* genes which encodes hexose transporters in *S. cerevisiae* by inactivating the transcriptional regulator Rgt1. The repression of the *HXT* genes enhanced cell growth rate (Kim, 2009; Lafuente et al., 2000; Moriya & Johnston, 2004a). Since the Mth1 mutation did not execute the de-repression of hexose transporters, the glucose uptake rate of the Mth1 mutants cannot be enhanced according to the external concentration of glucose. Although the internal deletion or point mutation of the *MTH1* gene in the TAM and SOS4 strains alleviated the cofactor imbalance induced by eliminating Pdc activity in the evolved Pdc-deficient strains, the glucose uptake rates of these strains were reduced (Kim et al., 2013a; Oud et al., 2012). As mentioned above, Pdc-deficient *S. cerevisiae* could not grow on glucose without the mutation on the *MTH1* gene. However, Pdc-deficient *S. cerevisiae* could grow on glucose when the 2,3-BD biosynthetic pathway enzymes are expressed (Kim et al., 2015; Nan et al., 2014). 1 mole of cytosolic NADH could be oxidized per 1 mole of 2,3-BD by the 2,3-BD biosynthetic pathway, and it gives the NADH accumulation and cofactor imbalance. Additionally, because α-acetolactate decarboxylase and α-acetolactate synthase have decarboxylation activity, a trace amount of pyruvate might be converted into acetaldehyde and

supplemented as C_2-compound to the Pdc-deficient *S. cerevisiae* strain (Atsumi et al., 2009).

In this chapter, 2,3-BD production of the engineered Pdc-deficient *S. cerevisiae* harboring the 2,3-BD biosynthetic pathway was evaluated. To identify the effect of *MTH1* mutation on 2,3-BD production, the fermentation performances of the 2,3-BD producing *S. cerevisiae* strains with *MTH1* mutation (BD4) and without *MTH1* mutation (BD5) were compared.

2.3. Materials and Methods

2.3.1. Strains and plasmids

Strains and plasmids used in this Chapter are summarized in Table 2-1 and primers are listed in Table 2-2. The *S. cerevisiae* D452-2 strain (*MATα*, *leu2*, *his3*, *ura3*, and *can1*) was used as a host strain for 2,3-BD production. For the introduction of the 2,3-BD biosynthetic pathway, two plasmids of pRS423_alsSalsD contained the *B. subtilis alsS* and *alsD* genes, and pRS425_BDH1 contained *S. cerevisiae BDH1* gene of which expression was under the control of the constitutive *TDH3* promoter and *CYC1* terminator. *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and manipulation.

2.3.2. Medium and culture conditions

E. coli was grown in Lysogeny Broth (LB) medium with 100 μg/mL ampicillin when required. Yeast strains were cultivated at 30°C in YP (10 g/L yeast extract, 20 g/L Bacto-peptone) or yeast synthetic complete (YSC) medium with 20 g/L glucose. To select transformants using an auxotrophic marker YSC medium containing 20 g/L glucose and 0.5 g/L ethanol was used. YSC medium contained 6.7 g/L yeast nitrogen base (YNB) and appropriate nucleotides and amino acids.

2.3.3. Yeast transformation and deletion of *PDC* gene

Transformation for introducing and overexpressing the genes involved in the 2,3-BD biosynthetic pathway in *S. cerevisiae* was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). Transformants were selected on YSC medium containing 20 g/L glucose and 0.5 g/L ethanol. Amino acids and nucleotides were added if necessary. The Pdc-deficient *S. cerevisiae* SOS2 strain (Kim et al., 2013a) was used as a host strain for the construction of the SOS5 strain. The *S. cerevisiae* SOS5 strain was constructed by deleting the *PDC6* gene in the SOS2 strain by a *URA3* marker recycling method as mentioned previously (Kim et al., 2013a) using the primers described in Table 2-2. The *B. subtilis alsS*, *alsD* and endogenous *BDH1* genes were expressed under the constitutive *S. cerevisiae TDH3* promoter. The *S. cerevisiae* BD5 and BD5-1 strain were constructed by transformation of p423_alsS_alsD, and p425_BDH1 plasmids and p426_alsS, p423alsD, and p425_BDH1 plasmids into the SOS5 strain, respectively.

2.3.4. Fermentation experiments

Seed cultures were prepared from cultivation on 5 mL of YSC medium containing 20 g/L glucose and 1 g/L ethanol. For preparation of pre-culture, cells in seed cultures were harvested and inoculated into 50 ml of YSC

medium with initial OD_{600} of of 1.0. For main batch fermentation, mid-exponential phase cells in pre-culture were harvested and inoculated into 50 mL of YSC medium with 90 g/L glucose without ethanol or 90 g/L glucose with 0.5 g/L ethanol in 250 mL flask at the OD_{600} of 1.0. Culture temperature of 30°C and agitation speed of 80 rpm were maintained throughout the fermentation experiments.

2.3.5. Analytical methods

Cell growth was monitored by optical density (OD) at 600 nm using a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and dry cell weight (DCW) was estimated by using a conversion factor: DCW (g/L) = OD * 0.2 (Kim et al., 2015). Glucose, glycerol, acetate, acetoin, 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. All reagents including (*R*, *R*)-2,3-BD for standard solution were purchased from Sigma-Aldrich.

2.3.6. Plasmid curing

For curing plasmids of the BD5-1 strain, cells were cultivated in YP medium containing 20 g/L ethanol. After 48 h of cultivation, grown cells were transferred into fresh YP medium containing 20 g/L ethanol on 5 ml test tubes and plates. The cells were serially subcultured until all of plasmids were cured. Each colony on plates was tested by the PCR reaction with primers in Table 2-2 to identify curing plasmids. The identified colonies were used for the next plasmid curing rounds.

2.4. Results

2.4.1. Comparison of 2,3-BD production by BD4 and BD5 strains

Batch fermentations were carried out for evaluating 2,3-BD production between the BD4 and BD5 strains in YNB medium containing 90 g/L of glucose without ethanol (Figure 2-1) and with 0.5 g/L of ethanol (Figure 2-2). Fermentation parameters are summarized in Table 2-3. The BD4 strain has a point mutation in the *MTH1* gene, and the BD5 strain has the wild type *MTH1* gene.

As shown in Figure 2-1, both BD4 and BD5 strains did not produce any ethanol because all structural *PDC* genes were deleted. The main product is 2,3-BD for both strains. Maximum DCW of the BD4 strain (2.2 g_{DCW}/L) was 5.5 times higher than that of the BD5 strain (0.4 g_{DCW}/L). Glucose consumption of the BD5 strain (26.9 $g_{Glucose}$/L) decreased to 53% that of the BD4 strain (50.2 $g_{Glucose}$/L) during 173.5 h of cultivation. 2,3-BD yields were similar for both strains (0.303 $g_{2,3\text{-}BD}/g_{Glucose}$ and 0.327 $g_{2,3\text{-}BD}/g_{Glucose}$), and glycerol yields were much higher in the BD5 strain (0.342 $g_{Glycerol}/g_{Glucose}$) than the BD4 strain (0.102 $g_{Glycerol}/g_{Glucose}$).

In the presence of 0.5 g/L ethanol in the fermentation medium, rates of cell growth and glucose consumption of both strains were substantially improved (Figure 2-2). Maximum DCW was similar for both strains (3.9 g_{DCW}/L for the BD4 strain and 4.2 g_{DCW}/L for the BD5 strain). Meanwhile,

the volumetric glucose consumption rate of the BD5 strain was substantially higher (1.23 $g_{Glucose}$/L/h) than that of the BD4 strain (0.66 $g_{Glucose}$/L/h). As a result, 2,3-BD productivity of the BD5 strain (0.40 $g_{2,3\text{-}BD}$/L/h) was 2-times higher than that of the BD4 strain (0.21 $g_{2,3\text{-}BD}$/L/h).

2.4.2. Plasmid curing of 2,3-BD-producing strain

To test an influence of the 2,3-BD biosynthetic enzymes on the viability and cell growth of Pdc-deficient *S. cerevisiae* strains, plasmid curing experiments were performed with the BD5 strain harboring *alsS*, *alsD*, and *BDH1* plasmids. As seen in Figure 2-3a, as subculture is repeated, the plasmids introduced were removed. After the 5th round of subculture, the strain without *alsS*, *alsD*, and *BDH1* plasmids was obtained. Plasmid curing efficiency of each subculture round was shown in Figure 2-3b. BDH1 plasmids were removed in most of colonies at the 2nd round of subculture. On the other hand, alsS and alsD plasmids were discarded at the 5th round of subculture.

2.5. Discussion

Mutation on the *MTH1* gene allows Pdc-deficient *S. cerevisiae* to grow on glucose as a sole carbon source (Kim et al., 2013a; Oud et al., 2012). Expression of the hexose transporter genes (*HXT*) was not induced by mutant *MTH1* strains (Polish et al., 2005). As a result, reduced glucose influx rates relieved cofactor imbalance of Pdc-deficient *S. cerevisiae*. Additionally, through a decreased intracellular concentration of glucose by reduced influx rates of glucose (Walsh et al., 1996; Ye et al., 1999). Accordingly, cell growth of the BD4 strain harboring the mutant *MTH1* gene was higher than that of the Pdc-deficient strains with the wild type *MTH1* gene. Thus, in the case of 2,3-BD fermentation under glucose as a sole carbon source (Figure 2-1), maximum DCW, glucose consumption rate, and 2,3-BD productivity of the BD4 strain could be much higher than the BD5 strain. On the other hand, in the presence of ethanol, the BD5 strain could synthesize acetyl-CoA from ethanol (Figure 2-2). In this condition, the maximum DCW of the BD5 strain was similar to that of the BD4 strain. Since the BD5 strain contains the wild type *MTH1* gene, hexose transporters could be induced by extracellular glucose. The induced transporter activity increased specific glucose uptake rates in the BD5 strain compared with the BD4 strain (Figure 2-4).

The 2,3-BD biosynthetic pathway is an NADH-consuming reaction. One mole of NADH is oxidized by one mole of 2,3-BD formed. The introduction

of the NADH-consuming 2,3-BD biosynthetic pathway could partially relieve the accumulation of cytosolic NADH in Pdc-deficient *S. cerevisiae* strains. Thus, as previously reported, Pdc-deficient *S. cerevisiae* strains could grow on glucose slowly as a sole carbon source by the introduction of the 2,3-BD biosynthetic pathway without the mutant *MTH1* gene (Kim et al., 2015; Nan et al., 2014). In the plasmid curing experiment, alsS and alsD plasmids remained until the 5th round of subculture and cured colonies were found on predominantly smaller colonies. It suggests the hypothesis that *alsS* and *alsD* could improve the growth of Pdc-deficient *S. cerevisiae*. The reason for the improved growth of Pdc-deficient *S. cerevisiae* by the introduction of *alsS* and *alsD* might due to the oxidation of additional NADH via the 2,3-BD biosynthetic pathway.

Table 2-1. Strains and plasmids used in Chapter 2.

Strains and plasmids	Description	Reference
Strains		
D452-2	*S. cerevisiae MATα, leu2, his3, ura3, can1*	In this study
SOS2	D452-2, *pdc1Δ pdc5Δ*	(Kim et al., 2013a)
SOS4	D452-2, *pdc1Δ pdc5Δ MTH1*^{G241C}	(Kim et al., 2013a)
SOS5	D452-2, *pdc1Δ pdc5Δ pdc6Δ*	In this study
BD4	SOS4, p423_alsSalsD, p425_BDH1	In this study
BD5	SOS5, p423_alsSalsD, p425_BDH1	In this study
BD5-1	SOS5, p426_alsS, p4253_alsD, p425_BDH1	In this study
Plasmids		
p426_alsS	*URA3 TDH3*$_{prom}$-*alsS-CYC1*$_{term}$	(Kim et al., 2013a)
p423_alsD	*HIS3 TDH3*$_{prom}$-*alsD-CYC1*$_{term}$	(Kim et al., 2013a)
p423_alsSalsD	*HIS3* 2μm origin *TDH3*$_{prom}$-*alsS-CYC1*$_{term}$ *TDH3*$_{prom}$-*alsD-CYC1*$_{term}$	(Kim et al., 2014)
p425_BDH1	*LEU2* 2μm origin *TDH3*$_{prom}$-*BDH1-CYC1*$_{term}$	(Kim et al., 2013a)

Table 2-2. Primers used in Chapter 2. 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**tactac gttatcgccgtgaattacgcaattcgcatgttacgattcg gtaatctccgaa
R_d_PDC6-4	***Xba*I**	GC**TCTAGA**tgtatttatttgtctgattgatttatgta ttcaaactgtggggtaataactgatataatta
R_d_PDC6-Check		gctgaacaacagtctctc
F_id_alsS		CGGGATCCATGTTGACAAAAGCA ACAAAAGA
R_id_alsS		CCGCTCGAGCTAGAGAGCTTTCG TTTTCA
F_id_alsD		CGGGATCCAAAATGAAACGAGAA AGCAACATTC
R_id_alsD		CCGCTCGAGTTATTCAGGGCTTCC TTCAG
F_id_BDH1		CGGGATCCAAAATGAGAGCTTTG GCATATTTC
R_id_BDH1		CCGCTCGAGTTACTTCATTTCACC GTGATTG

Table 2-3. Summary of batch fermentations by the BD4 and BD5 strains

Parameters		DCW $_{max}$ (g/L)	Glycerol (g/L)	2,3-BD (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
Without 0.5 g/L ethanol	**BD4**	2.2	5.1	15.2	0.102	0.303	0.09
	BD5	0.4	9.2	8.8	0.342	0.327	0.05
With 0.5 g/L ethanol	**BD4**	3.9	12.2	23.3	0.162	0.309	0.21
	BD5	4.2	21.6	27.6	0.248	0.324	0.40

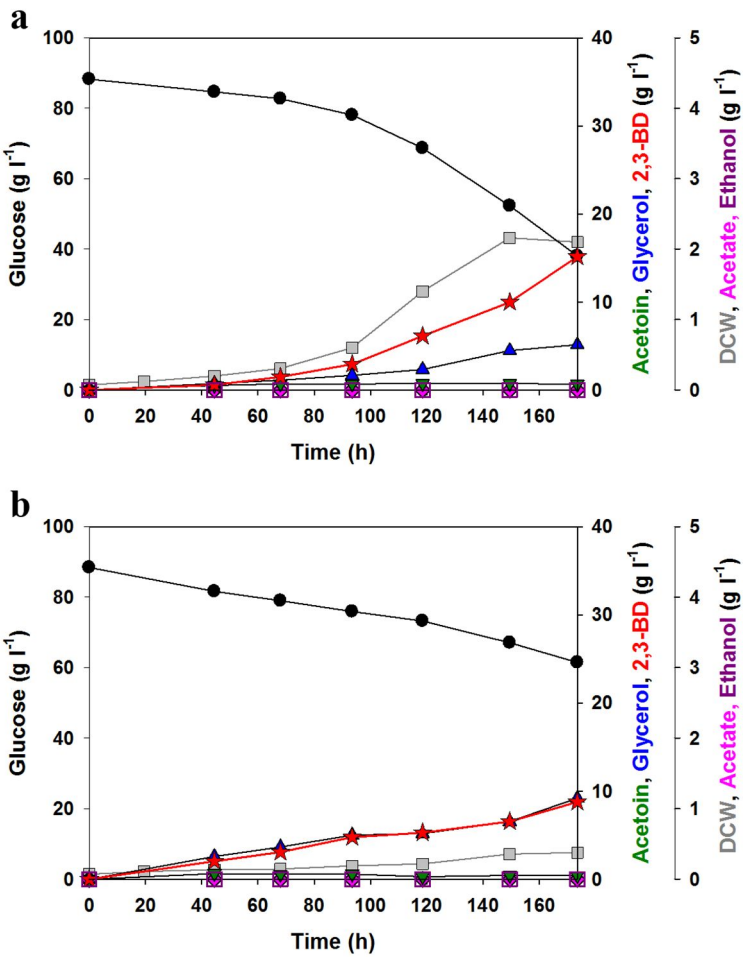


Figure 2-1. Batch fermentation of the (a) BD4 and (b) BD5 strains without ethanol. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

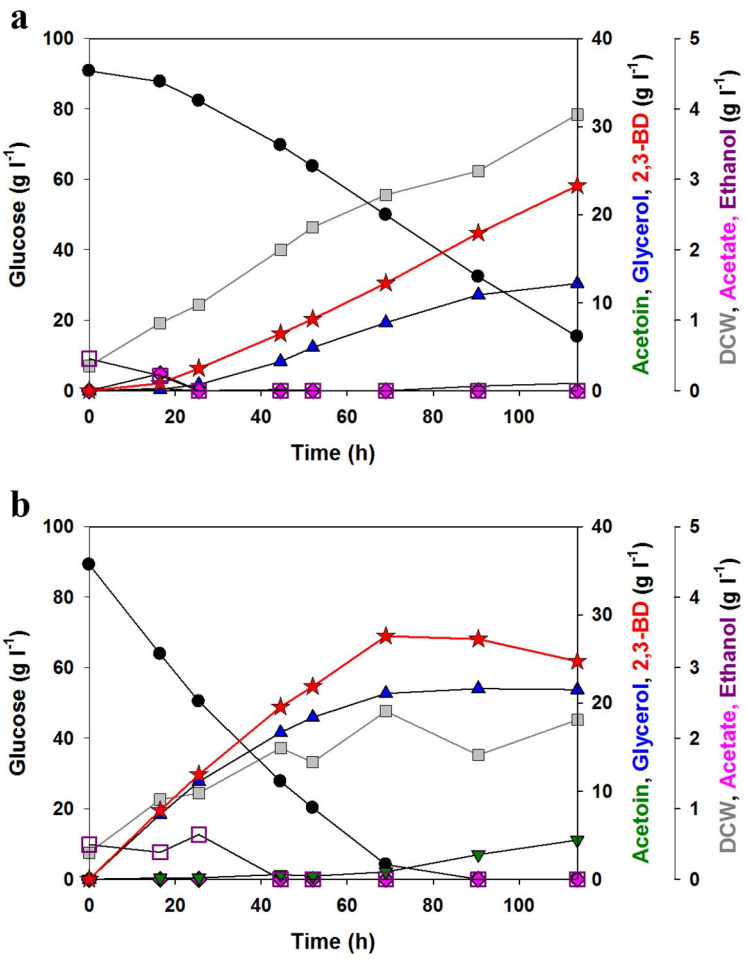
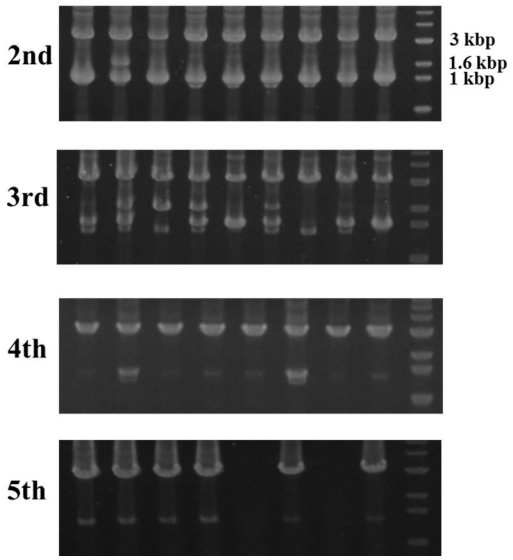


Figure 2-2. Batch fermentation of the (a) BD4 and (b) BD5 strains with ethanol. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

a



b

Gene	*alsS*		*alsD*		*BDH1*	
Colony size	Small	Large	Small	Large	Small	Large
2nd round	100%		100%		12.5%	
3rd round	100%	100%	87.5%	100%	8.3%	37.5%
4th round	95.8%	100%	4.2%	66.7%	0%	0%
5th round	25%	100%	0%	0%	0%	0%

Figure 2-3. Plasmid curing of the 2,3-BD strain. (a) Curing plasmids for expressing 2,3-BD production enzymes. 3.1 kbp, acetolactate synthase (*alsS*); 1.2 kbp, 2,3-butanediol dehydrogenase (*BDH1*); 0.8 kbp, acetolactate decarboxylase (*alsD*). (b) Efficiency of plasmid curing at each rounds. The number (%) means the probability of gene remained after plasmid curing.

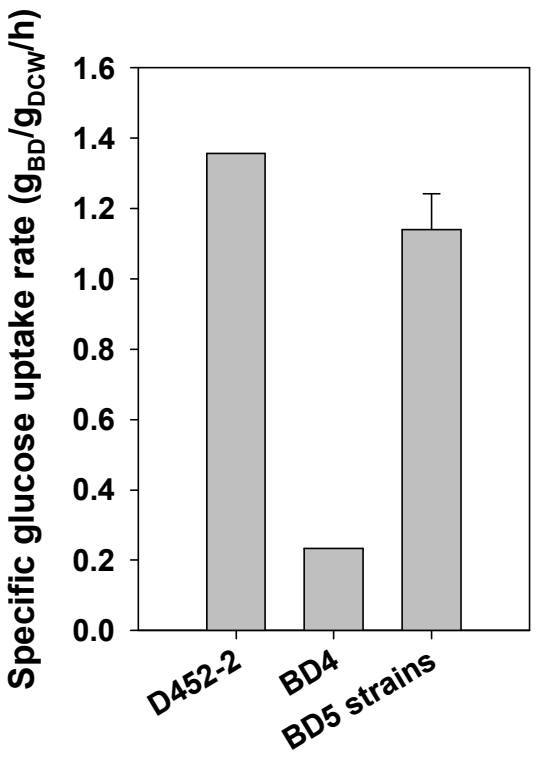


Figure 2-4. Specific glucose uptake rates of the D452-2, BD4, and BD5 strains.

Chapter 3.

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

3.1. Summary

In this chapter, the *B. subtilis* 2,3-BD biosynthetic enzymes (*alsS* and *alsD*), endogenous 2,3-butanediol dehydrogenase (*BDH1*), and *Lactococcus lactis* NADH oxidase (*noxE*) 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-compound supplementation. 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*. The expression of NADH oxidase substantially decreased the intracellular NADH/NAD^+ ratio. Production of 2,3-BD and glycerol by the BD5 strain were greatly influenced by the intracellular NADH/NAD^+ levels. The intracellular NADH/NAD^+ levels were controllable by dissolved oxygen (DO). The *S. cerevisiae* BD5_T2nox strain expressing *noxE* produced 2,3-BD with yield of 0.359 $g_{2,3\text{-}BD}/g_{Glucose}$ 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 NADH oxidase.

3.2. Introduction

2,3-Butanediol (2,3-BD) is a versatile chemical that has various applications to chemical industries. 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 & Chambers, 1987), and 1,3-butadiene can be used to make synthetic rubber (Syu, 2001; Winfield, 1945). Polyurethane forms can be made by the reaction of 2,3-BD and boric acid (Paciorek-Sadowska & Czupryński, 2006).

A number of studies on the production of 2,3- BD with bacterial strains such as *K. pneumoniae*, *K. oxytoca*, *E. aerogenes*, and *S. marcescens* have been reported (Petrov & Petrova, 2009; Qureshi & Cheryan, 1989; Zeng et al., 1991; Zhang et al., 2010). Alternatively, *S. 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 (Ostergaard et al., 2000). A wild-type *S. cerevisiae* produces trace amounts of 2,3-BD (0.4 to 2.0 g/L) during wine fermentation (Ehsani et al., 2009; Guymon & Crowell, 1967) although *S. cerevisiae* possesses three putative pathways converting pyruvate to 2,3-BD

(Romano & 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., 2013a; 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 reaction can be perturbed to eliminate ethanol production in yeast (Kim et al., 2013a). The engineered *S. cerevisiae* BY4742 *Δadh1Δadh3Δadh5* expressing *B. subtilis* acetolactate synthase (*alsS*), *E. 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., 2013a). 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).

Rerouting metabolic fluxes from ethanol to 2,3-BD formation resulted in the excess production of cytosolic NADH. 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. On the other hand, for the 2,3-BD biosynthesis, 2 moles of pyruvate produced by glycolysis were converted into 1 mole of 2,3-BD with only 1 mole of NADH oxidized as shown in Figure 3-1, (Syu, 2001). As a result, excess cytosolic NADH accumulates 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 dihydroxyacetonephosphate 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, as shown in Figure 3-2, 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., 2013a; Kim et al., 2014; Lian et al., 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 expression of ammonium assimilation enzymes (Nissen et al., 2000c), 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 (Geueke et al., 2003; Hoskins et al., 1962; Koike et al., 1985). Among them, *noxE* from *L. lactis* (Heux et al., 2006; Zhang et al., 2012a) 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 the 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 chapter, the gene for *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 the NADH oxidase gene 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.

3.3. Materials and Methods

3.3.1. Construction of plasmids

Strains and plasmids used in this study are summarized in Table 3-2. The primers used for cloning of *S. cerevisiae* promoters and NADH oxidase gene from *L. lactis* subsp. *cremoris* MG1363 are listed in Table 3-3. *E. 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 the genomic DNA of *S. cerevisiae* D452-2 using the primers in Table 3-3, and *S. cerevisiae TDH3* promoter (655 bp) was purified from p426GPD plasmids after *Sac*I and *Xba*I 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 3-3.

3.3.2. Yeast transformation and construction of recombinant *S. cerevisiae* strains

The Pdc-deficient *S. cerevisiae* SOS2 strain (Kim et al., 2013a) was used as a host strain for the construction of the engineered *S. cerevisiae* strains listed in Table 3-2. The *S. cerevisiae* SOS5 strain was constructed by deleting the *PDC6* gene in the SOS2 strain by the *URA3* marker recycling method as mentioned previously (Kim et al., 2013a) using the primers described in Table 3-3. 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^{o}C in YNB medium (6.7 g/L yeast 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 multicopy). The *L. lactis noxE* expression plasmids in Table 3-2 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 *Stu*I before use and integrated into the *URA3* locus. The resulting recombinant *S. cerevisiae* strains are listed in Table 3-2.

3.3.3. Fermentation conditions

All cultures were carried out at 30^{o}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 the 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^{o}C. The medium pH was maintained at 5.5 with 5N 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.

3.3.4. 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 x 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).

3.3.5. *In vitro* enzymatic assay of NADH oxidase

To prepare crude extracts, about 1 x 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 hours 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., 1998b). 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).

3.4. Results

3.4.1. Introduction of 2,3-BD biosynthetic pathway and 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 3-2). To express NADH oxidase in the 2,3-BD-producing *S. cerevisiae* strain, the NADH oxidase (*noxE*) gene from *Lactococcus lactis* subsp. cremoris was selected because of two reasons. First, *L. lactis* NADH oxidase has the least Km value among the reported NADH oxidases (Table 3-1). Second, the *L. lactis* NADH oxidase forms H_2O as a product after the reaction with NADH and molecular oxygen different from hydrogen peroxide (H_2O_2)-forming NADH oxidase (Lopez de Felipe & Hugenholtz, 2001). 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 3-2. The rationale of selecting the three promoters for altering expression levels of *noxE* are as follows. 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; Geertman et al., 2006).

The control and *L. lactis noxE* expressing plasmids (p426GPD, p406GPD2_nox, p406_TDH3_nox, p426CYC1_nox, p426GPD2_nox, p426_TDH3_nox) were introduced to the 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 3-2).

3.4.2. Differential NADH oxidase activities influenced intracellular NADH and NAD^+ concentration

To determine expression levels of NADH oxidase in engineered *S. cerevisiae* strains expressing *noxE* differently, NADH oxidase activities of the control and five *noxE* expressing strains were measured. As shown in Figure 3-3, 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, it can be speculated 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 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 almost 900-fold higher NADH oxidase activity of 9.15 U/mg protein than the BD5_G1nox strain (Figure 3-3).

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 3-4. 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 (Figure 3-4).

3.4.3. Production of 2,3-BD by 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 production by engineered *S. cerevisiae*, oxygen-limited cultures were carried out in minimal medium with 90 g/L glucose as a sole carbon source (Figure 3-5 and Table 3-5). 2,3-BD and glycerol were major products and acetoin was a minor product by the BD5_Con 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

those by 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 towards 2,3-BD and glycerol production were shifted toward acetoin production by the strong expression of NADH oxidase in the BD5_T2nox strain. The decreased cytosolic NADH/NAD^+ ratio in the BD5_T2nox strain (Figure 3-4) 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 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 maximum DCW and fermentation performance of 2,3-BD-producing Pdc-deficient *S. cerevisiae* were substantially reduced by expression of NADH oxidase (Figure 3-5b). 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 the *noxE*-expressing strains (Heux et al., 2006) might not be a reason for slow growth

and a poor fermentation performance of the engineered strains because acetaldehyde cannot be synthesized in Pdc-deficient *S. cerevisiae* strains. The higher acetoin accumulation and the 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.

3.4.4. Introduction of 2,3-BD biosynthetic pathway allows 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; Wang et al., 2012b). 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., 1999; Flikweert et al., 1996; van Maris et al., 2003). Furthermore, even in the presence of C_2-compounds, the insufficient regeneration of cytosolic NAD^+ reduced specific growth rate and glucose

consumption rate (Ishida et al., 2006; Kim et al., 2013a). As a result, the Pdc-deficient *S. cerevisiae* strains are unable to grow on defined media with glucose as a sole carbon source (Flikweert et al., 1996; Pronk et al., 1996). As recently reported, the mutation of *MTH1* allows the Pdc-deficient *S. cerevisiae* strains to overcome the growth defects on glucose (Kim et al., 2013a; Oud et al., 2012). Mth1p regulates glucose transporters by inactivating the transcriptional regulator Rgt1 (Moriya & Johnston, 2004b). 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 (Figure 3-5a), 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 partially 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).

3.4.5. 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, maximum DCW and glucose consumption rate were substantially lower than those of the wild type *S. cerevisiae*. The synthesis of cytosolic acetyl-CoA in the 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 the 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 the five NADH oxidase-expressing strains are shown in Figure 3-6. 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 the engineered strains expressing

different levels of NADH oxidase are compared in Table 3-6. When glucose was used as a sole carbon source in fermentation experiments, the BD5_T2nox strain showed substantially reduced maximum DCW and glucose uptake rates. Once ethanol is supplemented, however, the maximum DCW and glucose consumption rate of the BD5_T2nox strain significantly 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 to 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 ($g_{2,3\text{-BD}}/L \cdot h \cdot g_{DCW}$) increased for the strains expressing NADH oxidase. It is assumed that NAD^+ which is necessary for continued flux through glycolysis (Lunt & Vander Heiden, 2011), 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 while 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 (Figure 3-6). 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 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 much 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 by 10.7% compared to the BD5_Con strain. These results indicated that production of glycerol and 2,3-BD can be controlled by the 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.

The effect of NADH oxidase on product profiles during 2,3-BD fermentations were examined during batch fermentations in a bioreactor with the BD5_Con strain and BD5_T2nox strain which exhibited drastic phenotype changes in previous experiments (Figure 3-7). As previously reported (Heux et al., 2006), the BD5_T2nox strain expressing 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 (Figure 3-8). 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 the 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 $g_{2,3\text{-}BD}/g_{Glucose}$) was 23.8% higher than that of the BD5_Con strain (0.290 $g_{2,3\text{-}BD}/g_{Glucose}$). 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 from the excessive reaction of NADH oxidase of the BD5_T2nox strain in a bioreactor.

3.5. Discussion

During the early exponential phase (< 9 h), acetoin was rapidly produced in NADH oxidase expressing strains (Figure 3-9). In the BD5_T2nox strain, the highest acetoin (0.53 g/L) was produced among the engineered strains, which was a nine-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 (Figure 3-9). Despite of the large difference in acetoin concentration produced during the early exponential phase, the final yields of acetoin were similar for all tested strains and occupied only 1% of glucose consumed (0.009 to 0.012 g acetoin/g glucose) (Table 3-6). Acetoin can be produced from the reverse reaction of 2,3-BD by 2,3-butanediol dehydrogenase (*BDH1*) besides 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* strains. 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 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 a decreases of 2,3-BD (Figure 3-6). After glucose depleted (> 76 h), the generation of NADH by glycolysis and 2,3-BD production stopped. At this time, 2,3-BD might be oxidized to acetoin by the 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 3-6), the effects on 2,3-BD fermentation profiles were similar for 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 still exceeded 0.2 $g_{Glycerol}/g_{Glucose}$. This means that regeneration of cytosolic NAD^+ 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.

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, a 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 reactions unlike other cofactor engineering processes. Thus, the reaction of NADH oxidase could be simply regulated by protein expression levels combined with 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.

Table 3-1. Source of NADH oxidase and its kinetic constants.

Source	Km (uM)	Vmax (U/mg)	NADPH activity*	Host strain (Accession Number)	Reference
Lactobacillus brevis	24	116	No	-	(Hummel & Riebel, 2003)
S. pneumoniae	-	-	-	-	(Vemuri et al., 2007)
Lactococcus lactis	4.1	83.2	No	-	(Lopez de Felipe & Hugenholtz, 2001)
Leuconostoc mesenteroides	120	412	0.65%	-	(Koike et al., 1985)
Enterococcus faecalis	41	-	No	P37061 (SwissProt)	(Ross & Claiborne, 1992; Schmidt et al., 1986)
Bacillus cereus nox2(444)	200.8	22.9	No	-	(Wang et al., 2012a)
Lactobacillus rhamnosus	5.8	263	No	-	(Zhang et al., 2012b)
Clostridium aminovalericum	19.2	119	No	-	(Kawasaki et al., 2004)
Lactobacillus sanfrancisensis	6.1	39.3	28%	*E. coli*	(Riebel et al., 2003)
Borrelia burgdorferi	22.0	1.8	No	*E. coli*	(Riebel et al., 2003)
Mycoplasma pneumoniae	-	-	-	P75389 (SwissProt)	(Himmelreich et al., 1996)
Methanococcus japanicus	-	-	-	Q58065 (EMBL)	(Bult et al., 1996)
Bacillus cereus nox2(544)	23.6	2.4	No	-	(Wang et al., 2012a)
Thermococcus profundus	53.1	-	400%	*E. coli*	(Jia et al., 2008)
Streptococcus mutans	25	-	No	D49951 (EMBL)	(Matsumoto et al., 1996)

* % means capacity of activity with NADPH as a cofactor compared to NADH

Table 3-2. Strains and plasmids used in Chapter 3

Strains and plasmids	Description	Reference
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., 2013a)
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* 2μm origin	(Mumberg et al., 1995)
p426GPD	*URA3* 2μm 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* 2μm origin $TDH3_{prom}$-*alsS*-$CYC1_{term}$ $TDH3_{prom}$-*alsD*-$CYC1_{term}$	(Kim et al., 2014)
p425_BDH1	*LEU2* 2μm origin $TDH3_{prom}$-*BDH1*-$CYC1_{term}$	(Kim et al., 2013a)
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 3-3. Primers used in Chapter 3. Bold and capital characters are restriction enzyme sites.

Primers	Restriction site	Sequence
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

Table 3-4. The NADH and NAD^+ concentrations in engineered *S. cerevisiae* strains at 48 h under oxygen-limited conditions. Results are the averages of duplicate experiments with error bars.

Condition		Concentration ($\mu M/g_{DCW}$) (mean ± standard deviation)					
		BD5_ Con	BD5_ G1nox	BD5_ C2nox	BD5_ T1nox	BD5_ G2nox	BD5_ T2nox
80 g/L glucose	NADH	2.96 ± 0.16	2.48 ± 0.10	2.55 ± 0.06	1.83 ± 0.12	1.91 ± 0.06	1.90 ± 0.13
	NAD^+	9.47 ± 0.12	10.34 ± 0.07	11.02 ± 0.23	13.29 ± 0.50	12.94 ± 0.30	14.51 ± 0.90
80 g/L glucose 0.5 g/L ethanol	NADH	2.68 ± 0.05	2.40 ± 0.01	1.78 ± 0.03	1.23 ± 0.04	1.48 ± 0.01	1.44 ± 0.05
	NAD^+	8.61 ± 0.06	7.81 ± 0.17	7.57 ± 0.25	9.36 ± 0.05	8.99 ± 0.10	10.22 ± 0.00

Table 3-5. Fermentation parameters of engineered *S. cerevisiae* strains in oxygen-limited batch fermentation with a minimal medium containing 80 g/L glucose as a sole carbon source.

Parameters	DCW_{max} (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	Productivity (g/L/h)
BD5_Con	0.8	7.5	0.3	8.0	0.286	0.305	0.11
BD5_C2nox	0.2	5.0	0.8	4.7	0.287	0.270	0.07
BD5_T2nox	0.2	1.5	2.5	1.9	0.121	0.153	0.03

Table 3-6. 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*	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
$V_{glucose}$ ($g/L \cdot h^{-1}$)	1.10 ± 0.03	1.11 ± 0.02	1.06 ± 0.01	1.13 ± 0.00	1.07 ± 0.02	1.10 ± 0.00
$P_{2,3\text{-BD}}$ ($g/L \cdot h^{-1}$)	0.364 ± 0.011	0.374 ± 0.008	0.381 ± 0.003	0.404 ± 0.002	0.391 ± 0.006	0.403 ± 0.002

* 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/$L \cdot h^{-1}$); $P_{2,3\text{-BD}}$, 2,3-butanediol productivity (g 2,3-butanediol/$L \cdot h^{-1}$).

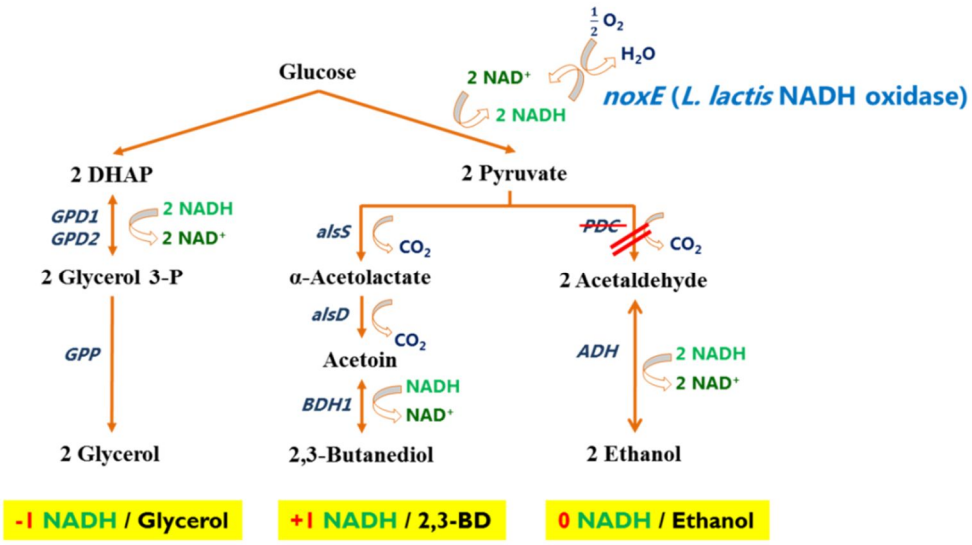


Figure 3-1. Cofactor balance of the recombinant 2,3-BD-producing Pdc-deficient *S. cerevisiae* strain.

(a) Anaerobic

$2C_6H_{12}O_6(Glucose) + 2NAD^+ \rightarrow 4CO_2 + 2C_4H_{10}O_2(2,3\text{-}BD) + 2NADH_2$

$C_6H_{12}O_6(Glucose) + 2NADH_2 \rightarrow 2C_3H_8O_3(Glycerol) + 2NAD^+$

$\underline{net,\ 3C_6H_{12}O_6(Glucose) \rightarrow 4CO_2 + 2C_4H_{10}O_2(2,3\text{-}BD) + 2C_3H_8O_3}$

Yield of 2,3-BDO = 0.333 g_{BDO}/g_{Glc} , Yield of Glycerol = 0.340 g_{Gly}/g_{Glc}

(b) Microaerobic

$4C_6H_{12}O_6(Glucose) + 4NAD^+ \rightarrow 8CO_2 + 4C_4H_{10}O_2(2,3\text{-}BD) + 4NADH_2$

$C_6H_{12}O_6(Glucose) + 2NADH_2 \rightarrow 2C_3H_8O_3(Glycerol) + 2NAD^+$

$O_2 + 2NADH_2 \rightarrow 2H_2O + 2NAD^+$

$\underline{net,\ 5\ C_6H_{12}O_6 + O_2 \rightarrow 8\ CO_2 + 4C_4H_{10}O_2(2,3\text{-}BD) + 2C_3H_8O_3 + 2H_2O}$

Yield of 2,3-BDO = 0.400 g_{BDO}/g_{Glc} , Yield of Glycerol = 0.204 g_{Gly}/g_{Glc}

(c) Aerobic

$C_6H_{12}O_6(Glucose) + NAD^+ \rightarrow 2CO_2 + C_4H_{10}O_2(2,3\text{-}BD) + NADH_2$

$½O_2 + 2NADH \rightarrow 2H_2O + 2NAD^+$

$\underline{net,\ C_6H_{12}O_6 + ½O_2 \rightarrow 2CO_2 + C_4H_{10}O_2(2,3\text{-}BD) + H_2O}$

Yield of 2,3-BDO = 0.500 g_{BDO}/g_{Glc} , Yield of Glycerol = 0 g_{Gly}/g_{Glc}

Figure 3-2. Theoretical yield of 2,3-BD production in *S. cerevisiae*. (a), Anaerobic; (b), Microaerobic; (c), Aerobic condition.

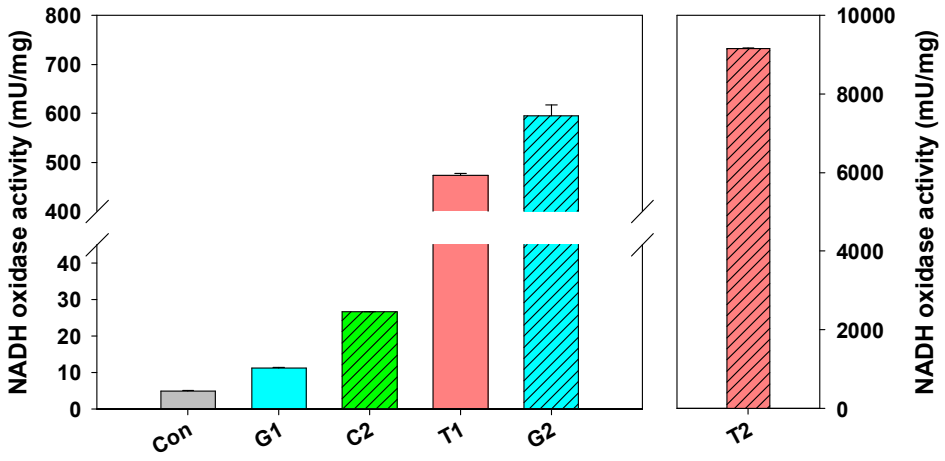


Figure 3-3. *In vitro* NADH oxidase activity in the control and five engineered BD5 strains expressing *noxE*. Con, the BD5_Con strain; G1, the BD5_G1nox strain; C2, the BD5_C2nox strain; T1, the BD5_T1nox strain; G2, the BD5_G2nox strain; T2, the BD5_T2nox strain. The exponentially growing cells in YNB containing 100 g/L glucose and 0.5 g/L ethanol were used for assay. One unit of activity was defined as the amount of enzyme oxidizing 1μ mol NADH per minute at the corresponding reaction conditions.

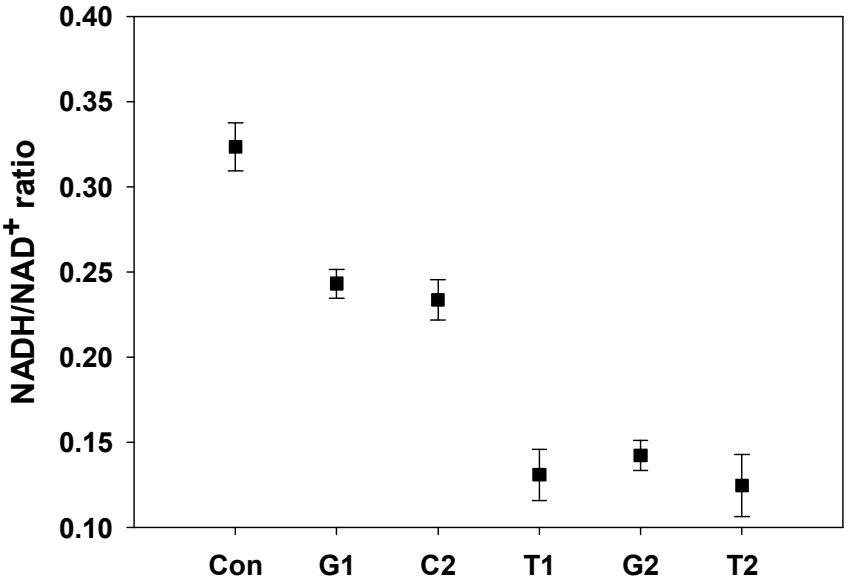


Figure 3-4. Intracellular NADH/NAD^+ ratio in *noxE* expressing *S. cerevisiae* strains under oxygen-limited conditions. Con, the BD5_Con strain; G1, the BD5_G1nox strain; C2, the BD5_C2nox strain; T1, the BD5_T1nox strain; G2, the BD5_G2nox strain; T2, the BD5_T2nox strain. Cells were harvested at 48 h from cultures using a minimal medium containing 90 g/L glucose. Results are the averages of duplicate experiments with error bars.

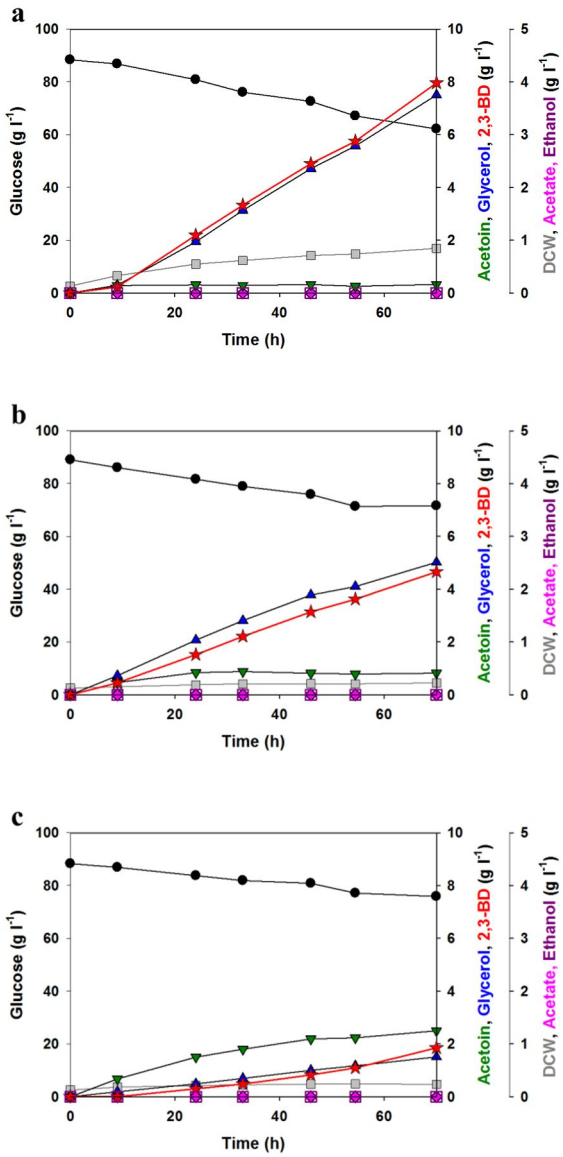
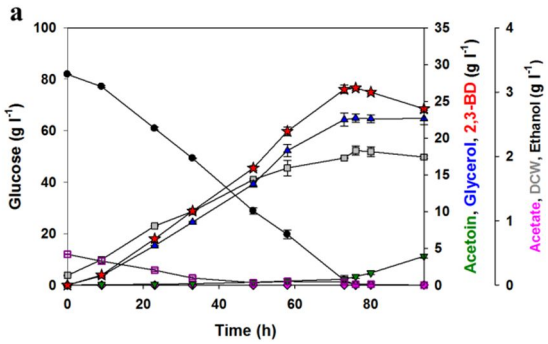
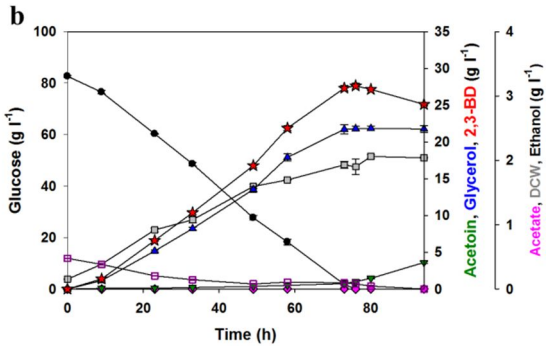
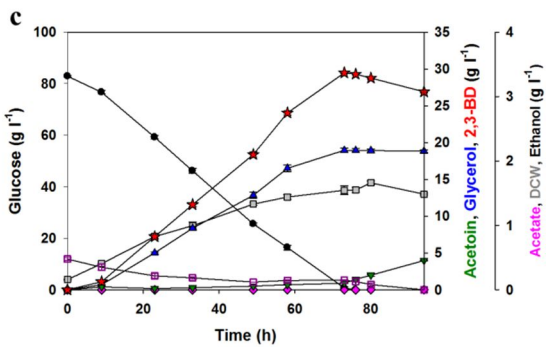
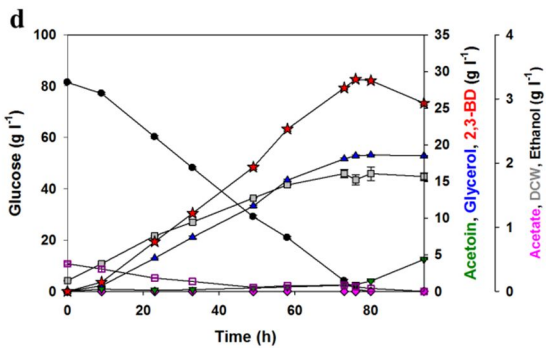


Figure 3-5. Flask cultivation of the control and NADH oxidase expressing strains without ethanol. (a) the BD5_Con strain, (b) the BD5_C2nox strain, (c) the BD5_T2nox strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.


a
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
Acetate, DCW, Ethanol (g l-1)
Time (h)


b
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
Acetate, DCW, Ethanol (g l-1)
Time (h)


c
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
Acetate, DCW, Ethanol (g l-1)
Time (h)


d
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
Acetate, DCW, Ethanol (g l-1)
Time (h)

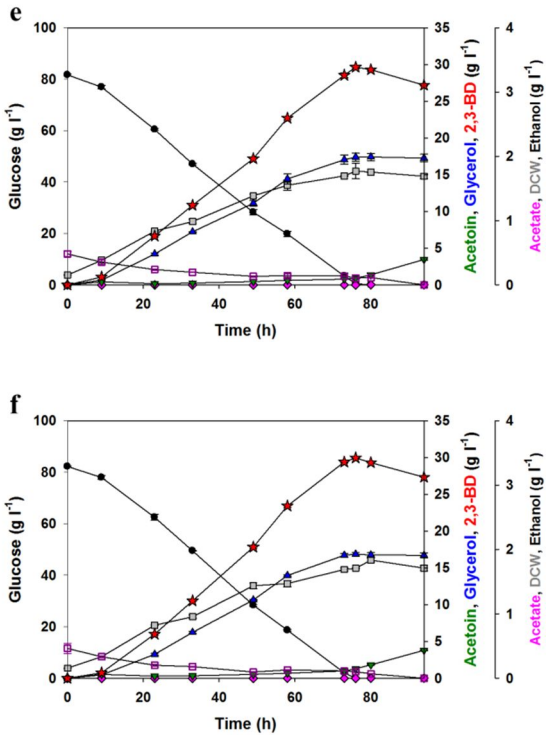


Figure 3-6. Flask cultivation of the control and NADH oxidase expressing strains with ethanol. (a) the BD5_Con strain, (b) the BD5_G1nox strain, (c) the BD5_T1nox strain, (d) the BD5_C2nox strain, (e) the BD5_G2nox strain, (f) the BD5_T2nox strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD. Results are the averages of duplicate experiments with error bars.

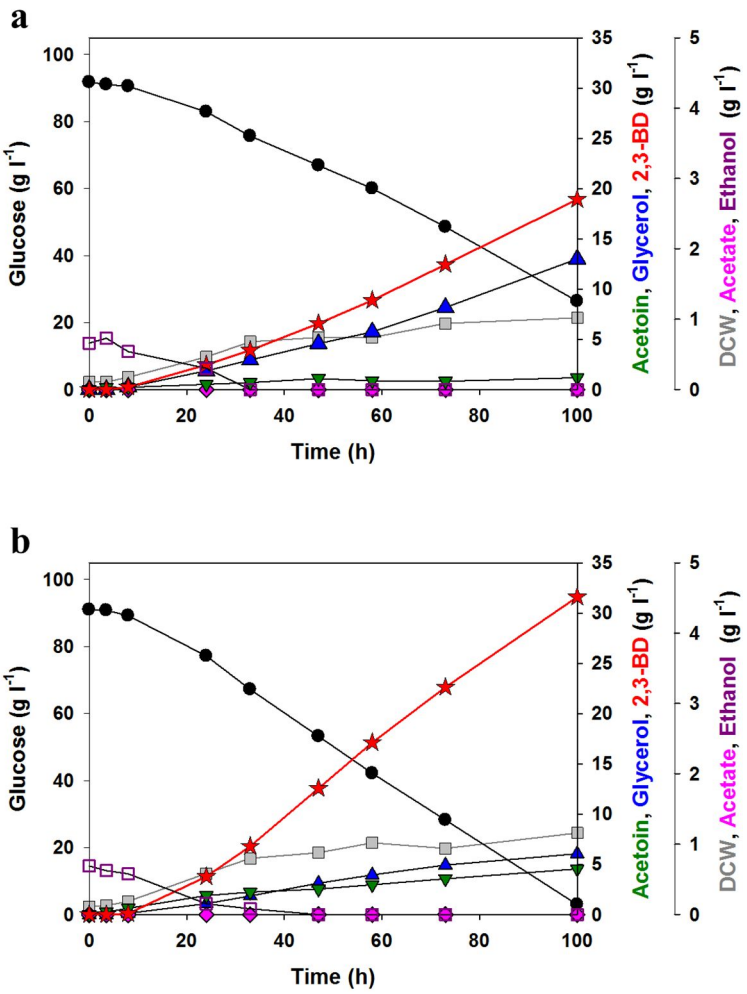


Figure 3-7. Fermentation profiles of the (a) BD5_Con and (b) BD5_T2nox strains in minimal medium with bioreactor. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

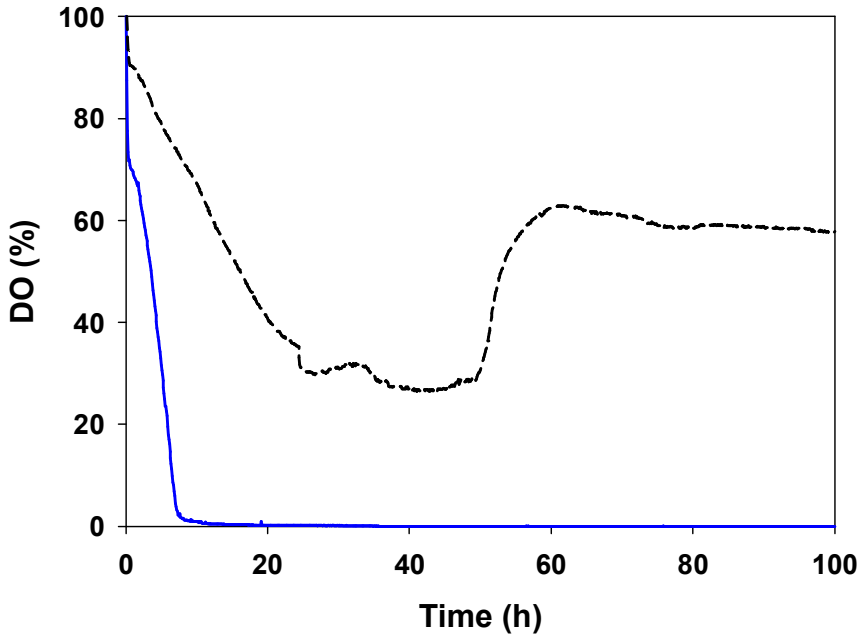


Figure 3-8. Dissolved oxygen (DO) levels of culture media during the batch cultivation. The values are relative oxygen levels as compared to saturated value (100%) in the medium. Symbols: BD5_Con (black, dashed), and BD5_T2nox (blue, solid)

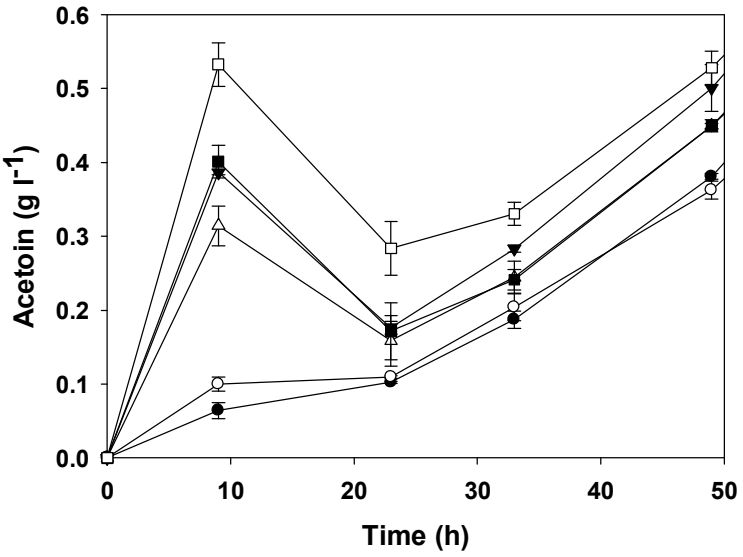


Figure 3-9. Effects of NADH oxidase expression levels on acetoin formation by engineered *S. cerevisiae* strains under oxygen-limited conditions. The early phase of fermentation (< 50 h) is highlighted from the data in Figure 4 and Figure S3. Symbols: BD5_Con (filled circle), BD5_G1nox (open circle), BD5_T1nox (inverted filled triangle), BD5_C2nox (open triangle), BD5_G2nox (filled square), and BD5_T2nox (open square). Results are the averages of duplicate experiments with error bars.

Chapter 4.

Fine-tuning *PDC* expression increases 2,3-butanediol productivity in Pdc-deficient *S. cerevisiae*

4.1. Summary

2,3-Butanediol (2,3-BD) is a promising compound that has various applications to chemical industry. Recently, Pdc-deficient *S. cerevisiae* has been used as a host strain for the production of 2,3-BD. To overcome a growth defect of Pdc-deficient *S. cerevisiae* on glucose, an evolved Pdc-deficient *S. cerevisiae* strain has been constructed by evolutionary engineering to grow on glucose as a sole carbon source (Kim et al., 2013a). However, the evolved Pdc-deficient *S. cerevisiae* strains still suffered from the low growth rate and substrate consumption rate on glucose because of inhibiting the expression of hexose transporters by mutation on the *MTH1* gene and lack of C_2-compounds to synthesize cytosolic acetyl-CoA. In this study, the Pdc-deficient *S. cerevisiae* harboring the wild *MTH1* gene was used as a 2,3-producing host strain. In addition, to relieve the C_2-auxotropy in Pdc-deficient *S. cerevisiae*, pyruvate decarboxylase (*PDC*) genes were introduced back. To maximize the 2,3-BD production, the expression levels of *PDC* were optimized by combination of several *PDC* genes of different sources, promoters and copy numbers. The resulting strain (BD5_G1CtPDC1) expressing *Candida tropicalis PDC1* by the *S. cerevisiae GPD2* promoter showed 2.8 folds higher productivity (0.28 g $_{2,3\text{-BD}}$/L·h) than that of the control strain (0.10 g $_{2,3\text{-BD}}$/L·h) which had no *PDC* genes. Through the fed-batch fermentation with the BD5_G1CtPDC1 strain, 121.8 g/L of 2,3-BD

was produced with 1.52 $g_{2,3\text{-}BD}$/L/h of productivity. In addition, the BD5_Ctnox strain coexpressing *CtPDC1* and *noxE* could produce 154.3 g/L of 2,3-BD in 78 h cultivation. The overall yield of 2,3-BD was 0.404 $g_{2,3\text{-}BD}/g_{Glucose}$ which is 80.7% of theoretical yield. Efficient production of 2,3-BD using Pdc-deficient *S. cerevisiae* with expression of the fine-tuned *PDC* and *noxE* is applicable to not only to 2,3-BD production but also other chemical production systems which require pyruvate as a precursor.

4.2. Introduction

There have been several reports about the production of 2,3-BD with engineered *S. cerevisiae* harboring the bacterial 2,3-BD biosynthetic enzymes (Kim et al., 2015; Kim et al., 2013a; Ng et al., 2012). By introduction of *B. subtilis* α-acetolactate synthase (*alsS*) and α-acetolactate decarboxylase (*alsD*), and overexpression of endogenous 2,3-butanediol dehydrogenase (*BDH1*), pyruvate could be converted into optically pure (2R,3R)- BD by *S. cerevisiae* strains (Kim et al., 2013a). In these studies, Pdc-deficient *S. cerevisiae* strains were adopted as a 2,3-BD production host (Kim et al., 2015; Kim et al., 2013a). Through deleting the *PDC* genes, 2,3-BD could be produced with high yield because pyruvate was used for the 2,3-BD production rather than ethanol production. However, the Pdc-deficient *S. cerevisiae* could not grow on glucose as a sole carbon source because of i) redox imbalance by accumulation of cytosolic NADH, and ii) lack of acetyl-CoA in cytosol which is required for synthesizing lysine and fatty acid (Flikweert et al., 1996). The mutation on *MTH1* allows Pdc-deficient *S. cerevisiae* strain to grow on glucose used as a sole carbon source. After introducing the 2,3-BD biosynthetic enzymes, the engineered strain with *MTH1* mutation could produce high titer of 2,3-BD from a fed-batch cultivation (Kim et al., 2013a). The mutation on *MTH1* decreased glucose influx rate because expression of the hexose transporter genes was not

induced by glucose (Oud et al., 2012). A slow glucose uptake rate by *MTH1* mutation might be responsible for suppression of the growth defect of Pdc-deficient strains on glucose by alleviating redox imbalance (Kim et al., 2013a). Additionally, although the exact mechanism was still unknown, the mutant *MTH1* could partially relieve the C_2-auxotropy of Pdc-deficient *S. cerevisiae* (Kim et al., 2013a; Oud et al., 2012). The mutation on *MTH1* was necessary for Pdc-deficient *S. cerevisiae* to grow on glucose as a sole carbon source, but reduced glucose consumption rate decreased 2,3-BD productivity in spite of high cell density cultivations (Kim et al., 2013a).

The Pdc-deficient *S. cerevisiae* strain with the wild *MTH1* gene is required to improve carbon consumption rate and 2,3-BD productivity. However, to overcome the growth defect on glucose of the Pdc-deficient *S. cerevisiae* strains, supplementation of C_2-compounds is necessary. In the Pdc expressing strains, acetyl-CoA could be synthesize from pyruvate via aldehyde dehydrogenase and acetyl-CoA synthetase. The excess expression of *PDC* produces a large amounts of ethanol instead of 2,3-BD. Therefore, expression levels of Pdc should be fine-tuned to balance between acetyl-CoA synthesis and supplementation of pyruvate for production of 2,3-BD.

In this study, the Pdc-deficient *S. cerevisiae* strain was engineered for efficient production of 2,3-BD from glucose. For the construction of C_2-independent strains without the *MTH1* mutation, *PDC* genes were expressed

for eliminating the C_2-auxotropy of 2,3-BD-producing Pdc-deficient *S. cerevisiae* strains. *PDC* expression levels were optimized for maximizing 2,3-BD productivity and yield through combination of the *PDC* gene sources, promoter and copy number. Then, *L. lactis* NADH oxidase was additionally expressed for increasing 2,3-BD yield because excess cytosolic NADH led to production of a large amount of glycerol as a by-product (Kim et al., 2015). The resulting engineered *S. cerevisiae* strain could produce 2,3-BD with high titer, productivity and yield comparable to the bacterial strains. Utilization of the engineered *S. cerevisiae* strain for 2,3-BD production could be an attractive alternative to bacterial systems for industrial applications.

4.3. Materials and Methods

4.3.1. Construction of plasmids

Plasmids used in this chapter are summarized in Table 4-1. The primers used for cloning of the *PDC* gene from various yeast species and *AUR1-C* gene are listed in Table 4-2. *E. coli* TOP10 (Invitrogen, Carlsbad, CA) was used for gene cloning and manipulation. *E. coli* transformants were grown in Lysogeny Broth (LB) medium with 100 μg/mL of ampicillin. To construct expression plasmids with different *PDC* genes, ORFs of *PDC* genes were amplified by PCR from genomic DNA of *C. tropicalis*, *K. marxianus*, and *S. cerevisiae* using the primers in Table 4-2. The amplified DNA fragments were ligated into appropriate restriction sites in p426GPD, p406CYC1, and p406GPD2 plasmids. In order to construct aureobasidin A resistance plasmids, the *AUR1-C* gene was amplified from pAUR101 plasmids by PCR with primers of F_SnaBI_AUR1-C and R_MfeI_AUR1-C. The amplified DNA fragments were ligated into the corresponding restriction sites in Table 4-2.

4.3.2. Yeast transformation and construction of recombinant *S. cerevisiae* strains

Transformation of plasmids for introducing *PDC* gene 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 nitrogen base and appropriate nucleotides and amino acid). 20 g/L of ethanol was used as a carbon source for culturing the SOS5 strain and 20 g/L of glucose and 1 g/L of ethanol for culturing the other strains. The *C. tropicalis PDC* gene was expressed under different native promoters (*TDH3*, *CYC1*, and *GPD2*) and copy number (single and multicopy). The *PDC* expression plasmids in Table 4-1 were introduced into the SOS5 and BD5 strains and the engineered *S. cerevisiae* strains expressing different levels of *PDC* were constructed. Integrative *PDC* expression plasmids (p406CYC1_CtPDC1, p406GPD2_CtPDC1, and p406TDH3_CtPDC1) were digested with *Stu*I before use and integrated into the *URA3* locus. The resulting recombinant *S. cerevisiae* strains are listed in Table 4-1.

4.3.3. 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 microaerobic conditions in 250 mL flasks at 80 rpm. In order to prepare inoculums, engineered *S. cerevisiae* cells were cultivated during for 48-72 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 YP medium contains 100 g/L glucose and YNB medium contains 90 g/L glucose and 50 mM potassium phthalate at pH 5.5 adjusted by NaOH. Ethanol 0.5 g/L was added if necessary.

Fed-batch fermentations with bioreactor were carried out in 500 ml YP medium containing 300 g/L glucose using 1 L-bench-top fermentor (Fermentec, Korea) at 30^{o}C. The medium pH was maintained at 5.5 with 5N NaOH solution and dissolved oxygen (DO) levels were monitored with O_2 sensor (Mettler Toledo, Switzerland). The culture medium was agitated at 300-500 rpm and aerated with air flow rate of 1-2 vvm according to DO levels in medium. During the fed-batch fermentation, DO levels were kept under 2.0. The grown cells prepared from flask culture were inoculated at the initial concentration of 2.0 g/L.

4.3.4. 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, 2,3-BD, and ethanol were analyzed by a high-performance liquid chromatography (1100 series, Agilent, CO) equipped with a Rezex ROA-organic acid column (Phenomenex, CA). Metabolites were detected by a refractive index (RI) detector.

4.3.5. *In vitro* Pdc activity analysis

To prepare crude extracts, about 1 x 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 (Y-PER, Thermo Scientific, MA). After centrifugation for 20 min at 12,000 rpm and 4^{o}C, the supernatants were used to determine the Pdc activity within 3 h and diluted with DDW if necessary. The NADH oxidase activity assays were performed at 30^{o}C with the reaction mixture containing 40 mM imidazole hydrochloride buffer (pH 6.5), 5 mM $MgCl_2$, 0.2 mM TPP, 10U alcohol dehydrogenase from *S. cerevisiae*, 0.4 mM NADH, and 50 mM pyruvate (Postma et al., 1989). The reactions were initiated by adding pyruvate, 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).

4.3.6. Sample preparation for metabolite analysis

For the extraction of metabolome sampling, a fast filtration method was performed with a slight modification of the method used in a previous study (Kim et al., 2013b). Briefly, cell pellets on each conditions were collected by vaccum-filtering of 1 mL culture broth using a nylon membrane filter (0.45 μm pore size, 30 mm diameter; Whatman, Piscataway, NJ), and washed using 5 mL of distilled water at room temperature. The membrane filter and loaded cells were mixed using 10 mL of acetonitrile/water (1:1, v/v) at -20^{o}C. After that, the extraction mixture was immersed in liquid nitrogen. These steps were completed less in 30 s. The extraction mixture was thawed on ice, vortexed for 3 min, and centrifuged at 16,100 x g for 5 min at 4^{o}C. The supernatant was collected and vacuum-dried.

4.3.7. GC/TOF MS analysis

For the GC/TOF MS analysis, the derivative metabolite samples were prepared using 5 μL of 40 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich, St. Louis, MO) at 30^{o}C for 90 min and 45 μL of N-methyl-N-trimethylsilyl trifluoroacetamide (Fluka, Buchs, Switzerland) at 37^{o}C for

30 min. As retention index markers, a mixture of fatty acid methyl esters was added to the derivative samples. An Agilent 7890A FX (Agilent Technologies, Wilmington, DE) coupled with a Pegasus HT TOF MS (LECO, St. Joseph, MI) was used for metabolite analysis. A 0.5 μL of derivative samples was injected into the GC in splitless mode and separated on an RTX-5Sil MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA) with an additional 10 m integrated guard column. The initial oven temperature was set at 50°C for 1 min and then increased to 330°C at a rate of 20°C/min and held at 330°C for 5 min. Mass spectra were recorded in the mass range of 85-500 *m/z* at an acquisition rate of 10 spectra/s. The temperatures of the ion source and transfer line were set at 250°C and 280°C, respectively, and the sample ionization was performed electron impact at 70 eV.

4.3.8. Data processing and statistical analysis

For the processing of GC/TOF MS data, the software LECO Chroma TOF was used to detect peaks and to deconvolute the mass spectra. The processed data were further processed using an inhouse library, BinBase (Lee & Fiehn, 2008). The raw metabolite data were normalized by the median of the sum of the peak intensities of all of identified metabolites in each sample. The normalized data were used for multivariate and univariate statistical analysis

(Denkert et al., 2008), and MultiExperiment Viewer was used for the hierarchical clustering analysis (HCA).

4.4. Results

4.4.1. Selection of a suitable *PDC* gene for fine-tuning expression levels

The mutation on *MTH1* is regarded as an efficient strategy for improving 2,3-BD production by *S. cerevisiae* because the mutation on *MTH1* could resolve the C_2-compound dependent growth (C_2-auxotrophy) of Pdc-deficient *S. cerevisiae* strains (Kim et al., 2013a; Oud et al., 2012). However, the mutation on the *MTH1* gene reduced expression levels of hexose transporters (Flick et al., 2003; Kim et al., 2006). Thus, the mutation on *MTH1* could decrease 2,3-BD productivity because of the reduced carbon utilization rates.

The expression of *PDC* is a simple way to supply acetyl-CoA to the Pdc-deficient *S. cerevisiae*, however, the expression levels of *PDC* should be fine-tuned to maximize 2,3-BD production. In the previous report, less than 0.60 g/L of 2,3-BD was produced because a predominant carbon flux of pyruvate into ethanol in spite of partial deletion of alcohol dehydrogenase (Ng et al., 2012). As mentioned in Chapter 2, maximum DCW and glucose consumption rate of 2,3-BD-producing strains was significantly improved when a small amount of ethanol (0.5 g/L) was added in fermentation medium. Appropriate control of Pdc activity could be an efficient way to improve cell growth and 2,3-BD production in the Pdc-deficient 2,3-BD-producing *S. cerevisiae*. Therefore, to optimize Pdc- expression levels, the activity from

the expression of several *PDC* genes was measured in Pdc-deficient *S. cerevisiae*. Among the three *PDC* structural genes in *S. cerevisiae*, *PDC1* and *PDC5* were functionally expressed (Hohmann, 1991). The *PDC* activity of wild type *S. cerevisiae* is almost exclusively the product of *PDC1*, but the *PDC1* deletion mutant retains 80% of the specific wild type activity by auto-regulation (Hohmann & Cederberg, 1990). The metabolic characteristics of Crabtree-positive and negative yeasts are determined by the differences in the pyruvate metabolism through *PDC* activity (De Deken, 1966; Postma et al., 1989; van Urk et al., 1990). The Pdc activities of the Crabtree-negative yeasts were lower than the the Crabtree-positive yeasts, and did not increase in spite of the addition of excess glucose (van Urk et al., 1990). Therefore, the *PDC* genes from *C. tropicalis* and *K. marxianus* which are Crabtree-negative yeasts (De Deken, 1966; van Urk et al., 1990) were selected as candidate genes and its kinetic constants were compared with those of endogenous *PDC1*, *PDC5* and *PDC6* in the Pdc-deficient *S. cerevisiae* strain (Table 4-3). As a result, the *C. tropicalis PDC1* gene (*CtPDC1*) showed the lowest Pdc activity among the five genes tested. The *CtPDC1* gene was selected to express *PDC* in the 2,3-BD-producing strain. The Ctpdc1 and Scpdc1 proteins present 66% identical amino acid residues. Additionally, both Ctpdc1 and Scpdc1 proteins retain all structurally important amino acid residues for the Mg^{2+} and thiamine pyrophosphate (ThDP) binding loop

(N473, N474, G476, D446, T392, S448, I479) and substrate activation (C223) that are conserved throughout all *PDC*s (Arjunan et al., 1996; Baburina et al., 1994; Pohl, 1997) (Figure 4-1).

4.4.2. Optimization of *PDC* expression levels for efficient 2,3-BD production

To maximize 2,3-BD production by optimizing *PDC* expression levels in the Pdc-deficient *S. cerevisiae*, the *CtPDC1* gene were expressed with combination of promoters and copy numbers in the BD5 strain (Table 4-1). Then, *in vitro* Pdc activities in the control and Pdc-expressed strains were measured (Figure 4-2). As a result, the Pdc activity of the BDt_C1CtPDC1, BD5_G1CtPDC1, BD5_C2CtPDC1, and BD5_T2CtPDC1 strains were 74.7, 122.1, 287.4, and 1387.0 mU/mg protein respectively.

The wild type D452-2 and engineered strains were cultivated in complex medium (Figure 4-3, Table 4-4) containing 100 g/L glucose to explore how the differences of Pdc activity could influence the performance of 2,3-BD production. In batch fermentation with YP complex medium, the maximum DCW and glucose consumption rate of the engineered Pdc-deficient *S. cerevisiae* strains were much lower than the D452-2 wild type strain. The maximum DCW of the D452-2 strain (7.1 g/L) was 5.9-fold higher than that of the BD5_CtCtPDC1 strain. As the maximum DCW increased according to

the the Pdc activities, the BD5_T2CtPDC1 strain showed maximum DCW (6.5 g/L) similar to the D452-2 strain. The cell growth rates influenced the volumetric glucose consumption rates. The glucose consumption rate of the BD5_G1CtPDC1 strain (2.88 $g_{Glucose}$/L/h) was 54% for that of the D452-2 strain. Batch fermentations were carried out to compare the 2,3-BD production among the engineered strains in minimal medium containing 90 g/L glucose (Figure 4-4, Figure 4-5). The maximum DCW and glucose consumption rate were improved by the expression of *PDC* on glucose minimal medium regardless of the levels of Pdc activity. The maximum DCW of the BD5_G1CtPDC1 strain which has the second Pdc activity among the engineered strains increased by a 2-fold compared to the control strain. While the BD5_G1CtPDC1 strain consumed 95 g/L glucose completely in 100 h, the control strain consumed only 40 g/L of glucose. The engineered strains showed different product profiles according to the *PDC* expression levels. Contrary to the BD5_T2 CtPDC1 strain which produced a large amount of ethanol, the BD5_G1CtPDC1 strain produced less than 0.5 g/L of ethanol from 92.0 g/L glucose. Thus, the BD5_G1CtPDC1 strain could produce 2,3-BD with minimization of ethanol production to synthesize acetyl-CoA in the Pdc-deficient *S. cerevisiae* strain. As a result, the BD5_G1CtPDC1 strain was the best strain that showed the highest productivity among the engineered 2,3-BD-producing strains. The 2,3-BD

productivity of the BD5_G1CtPDC1 strain (0.28 $g_{2,3\text{-}BD}$/L/h) was 2.8-folds higher than that of the BD5_Con strain (0.10 $g_{2,3\text{-}BD}$/g/L).

4.4.3. Additional expression of NADH oxidase increased 2,3-BD yield and productivity

The reason for production of a large amount of glycerol is excess NADH formation in cytosol by 2,3-BD-producing Pdc-deficient *S. cerevisiae* (Kim et al., 2015). Thus, for reducing glycerol production, NADH oxidase was additionally expressed in the BD5_G1CtPDC1 strain to construct the BD5_Ctnox strain. The expression of NADH oxidase in *S. cerevisiae* could suppress glycerol formation by reducing intracellular NADH/NAD^+ levels (Kim et al., 2015). The batch cultivations with the BD5_Ctnox strain were performed for evaluating the effect of NADH oxidase on 2,3-BD fermentation in low (25%), medium (50%), and high (100%) aeration conditions. As a result, the yields of fermentation products were significantly changed according to the levels of aeration (Figure 4-6). At high aeration condition (100% air), glycerol yield of the BD5_Ctnox strain was only 1.3% of that of the BD5_G1CtPDC1 strain. 2,3-BD yields increased at the low and medium aeration conditions (0.358 and 0.374 $g_{2,3\text{-}BD}/g_{Glucose}$) in contrast to the high aeration condition (0.207 $g_{2,3\text{-}BD}/g_{Glucose}$) which produced a large amount of acetoin (0.224 $g_{Acetoin}/g_{Glucose}$) (Figure 4-7).

4.4.4. *In vivo* activity of NADH oxidase influence on intracellular metabolites

Cofactor imbalance induced by deletion of *PDC* genes in *S. cerevisiae* was relieved by NADH oxidase. To explain the effect of NADH oxidase on metabolite levels, analysis of intracellular metabolites was performed from batch fermentations with the BD5_G1CtPDC1 and BD5_Ctnox strains (Figure 4-8). The batch fermentations were carried out in different conditions including aerobic cultivation of the BD5_G1CtPDC1 strain (N0), microaerobic (N50) and aerobic (N100) cultivation of the BD5_Ctnox strain. It is assumed that *in vivo* NADH oxidase activity is changed by aeration conditions. As a result, product yields were significantly changed similar with results in Figure 4-6 and Figure 4-7. The glycerol yield was reduced and acetoin yield were increased according to the *in vivo* activity of NADH oxidase. Moreover, glucose consumption rate (2.39 $g_{Glucose}$/L/h) of the N0 condition was improved to 4.19 $g_{Glucose}$/L/h for the N50 condition and 4.30 $g_{Glucose}$/L/h for the N100 condition. In addition, 2.4 g/L of maximum DCW for the N0 condition was also increased to 4.5 g/L (N50) and 5.2 g/L (N100) (Figure 4-8). Intracellular metabolites in these conditions were analyzed to explain the higher maximum DCW and glucose consumption rate in the BD5_Ctnox strain than the BD5_G1CtPDC1. Cells were harvested at 22 h and total 129 intracellular metabolites were identified and quantified

(Appendix 1). These metabolites belonged to different chemical classes including amino acid, organic acids, sugars and sugar alcohols, fatty acids, phosphates, amines, nucleotides and others. Multivariate data were analyzed by principal component analysis (PCA) and hierarchical clustering analysis (HCA) to explore the variations of intracellular metabolites by *in vivo* NADH oxidase activity (Figure 4-9 and Figure 4-10). As shown in Figure 4-9, samples from each conditions (N0, N50, and N100) were clearly separated by PCA. It indicated that in the existence and *in vivo* activity of NADH oxidase changed intracellular metabolism and displayed distinctively metabolic characteristics. Intermediates of glycolysis, TCA cycle, and amino acid synthesis were significantly affected by NADH oxidase (Figure 4-11). First, the concentrations of glucose, and glucose-6-phosphate, and pyruvate which are the intermediates of glycolysis were up-regulated in the BD5_G1CtPDC1 strain (N0) compared with the BD5_Ctnox strain (N50). Pyruvate production from glucose by glycolysis is NADH-generating process because two moles of NADH are formed at glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction per one mole of glucose (Figure 4-11). Since cofactor imbalance by reduced oxidation of cytosolic NADH inhibited glycolytic flux and cell growth rate in Pdc-deficient *S. cerevisiae* (Kim et al., 2013a; Oud et al., 2012), the inhibition of glycolytic flux in the BD5_G1CtPDC1 strain might accumulate glycolytic

intermediates such as glucose, glucose-6-phosphate, and pyruvate. Metabolic reactions with isocitrate and fumarate as substrate are NADH-generating steps in TCA cycle. As similar with glycolytic intermediates, these compounds might be accumulated because of intracellular cofactor imbalance of NADH in the BD5_G1CtPDC1 strain (N0). From the analysis of intracellular metabolites in the BD5_G1CtPDC1 and BD5_Ctnox strains, the relieved cofactor imbalance by NADH oxidase changed glycolytic intermediates as well as TCA cycle intermediates. These data could propose hypothesis to explain the improved maximum DCW and glucose consumption rate by the expression of NADH oxidase in Pdc-deficient *S. cerevisiae*.

4.4.5. Fed-batch fermentation of the engineered strains expressing *PDC* and *noxE*

To evaluate the 2,3-BD production performance of the BD5_G1CtPDC1 strain, fed-batch fermentation was carried out with glucose as a sole carbon source (Figure 4-12). Initial 1.98 g/L of cell mass increased to 3.90 g/L in 80 h cultivation. Finally, 121.8 g/L of 2,3-BD was produced with productivity of 1.52 $g_{2,3\text{-BD}}$/L/h which is 3.9-folds higher than the fermentation with the BD4 strain (0.39 $g_{2,3\text{-BD}}$/L/h) in the previous report (Kim et al., 2013a). While small amounts of ethanol and acetoin (<1.0 g/L) were produced, a

large amount of glycerol (118.5 g/L) was produced as by-products. A yield of glycerol (0.333 $g_{Glycerol}/g_{Glucose}$) was similar to a yield of 2,3-BD (0.337 $g_{2,3\text{-}BD}/g_{Glucose}$).

Fed-batch fermentation was carried out to test whether the BD5_Ctnox strain could be a promising host strain for producing 2,3-BD (Figure 4-13). Aeration conditions were controlled with two stages for maximizing 2,3-BD by minimizing by-products formation according to the results of batch fermentation (Figure 4-6). In the first stage, fermentation medium was fully aerated for suppressing glycerol formation. 16.7 g/L of acetoin was produced as a by-product with 97.9 g/L of 2,3-BD until 42.3 h of cultivation. After that, when the fermentation medium was under oxygen-limited condition in the second stage, the produced acetoin was converted into 2,3-BD. As a result, final 154.3 g/L of 2,3-BD was produced with 1.98 $g_{2,3\text{-}BD}$/L/h of productivity. The overall yield of 2,3-BD was 0.404 $g_{2,3\text{-}BD}/g_{Glucose}$ which is 80.7% of theoretical yield. Glycerol yield (0.088 $g_{Glycerol}/g_{Glucose}$) of the BD5_Ctnox strain decreased 73.6% compared with that of the BD5_G1CtPDC1 strain. Final cell mass of 6.1 gDCW/L 1.6-folds increased compared with the BD5_G1CtPDC1 strain. Acetate was produced after 30 h of cultivation. The final acetate concentration was 2.3 $g_{Acetate}$/L and yield was 0.006 $g_{Acetate}/g_{Glucose}$. The parameters of fed-batch fermentations are summarized in Table 4-5.

4.5. Discussion

The goal of the present study was to engineer *S. cerevisiae* to produce 2,3-BD with high yield and productivity. The deletion of pyruvate decarboxylase could be a promising strategy for improving 2,3-BD yield of *S. cerevisiae* because pyruvate is the branch point to produce ethanol and 2,3-BD (Kim et al., 2015; Kim et al., 2013a). Cytosolic acetyl-CoA is an indispensable compound for growth of *S. cerevisiae* because acetyl-CoA is used to synthesize lysine and fatty acids in cytosol (Flikweert et al., 1999; Flikweert et al., 1996; Pronk et al., 1996). Pdc-deficient *S. cerevisiae* cannot synthesize cytosolic acetyl-CoA because acetaldehyde which formed by a reaction of pyruvate decarboxylase with pyruvate is a main precursor for acetyl-CoA synthesis in cytosol. In mitochondria, the *PDH* complex converts pyruvate into acetyl-CoA, but mitochondrial acetyl-CoA cannot pass the inner membrane of mitochondria (Van Roermund et al., 1995). Although the YBR219C and YBR220C are putative genes for acetyl-CoA transport, activity of these enzymes was low to supply enough acetyl-CoA (Giaever et al., 2002). Therefore, cell growth and carbon utilization of the Pdc-deficient *S. cerevisiae* strains were greatly inhibited because of an insufficient supplementation of cytosolic acetyl-CoA called a C_2-auxotropy. In the previous study, to overcome the growth defect of the Pdc-deficient *S. cerevisiae* strain, the evolved strain (BD4) that has a point mutation on the

MTH1 gene was used as a 2,3-BD-producing strain (Kim et al., 2013a). The evolved strain overcame the C_2-auxotropy, but led to reduced expression levels of hexose transporters (Kim, 2009; Lafuente et al., 2000; Moriya & Johnston, 2004a) and reduced carbon utilization rate as described in Chapter 2. Thus, the Pdc-deficient *S. cerevisiae* strain was engineered by additional expression of *PDC* and the C_2-auxotropy could be successfully alleviated without mutation on *MTH1* because acetyl-CoA was supplied by the expression of *PDC*. Pyruvate is a precursor not only for the synthesis of acetyl-CoA, but also for 2,3-BD production.

As shown in Fig. 4-5, the product yields and 2,3-BD productivities were changed according to the levels of Pdc activity. The BD5_Con strain produced glycerol and 2,3-BD with similar yields because of NADH balance between glycerol and 2,3-BD production described in Chapter 3. As the increased Pdc activity, ethanol production was increased, and even the BD5_T2CtPDC1 strain produced ethanol as predominantly rather than 2,3-BD production. In addition, the production of ethanol by additional *PDC* expression resulted in reduced glycerol production because cofactor imbalance might be relieved by ethanol biosynthesis which is a redox neutral process. The BD5_G1CtPDC1 strain was the most efficient 2,3-BD production strain because ethanol production was minimized along with C_2-compound supplementation for cell growth. On the other hand, the

BD5_T2CtPDC1 strain could be used as an efficient host strain for dual production of 2,3-BD and ethanol because of minimized glycerol production. As a result, through fine-tuning expression levels of Pdc, C_2-compounds were efficiently supplied for synthesizing acetyl-CoA and 2,3-BD or ethanol production could be easily modulated. The fine-tuned expression of *PDC* could be applied to improve other chemical production systems using pyruvate as a precursor compound.

Table 4-1. Strains and plasmids used in Chapter 4

Strains and plasmids	Description	Reference
Strains		
Candida tropicalis ATCC20336	Source for *CtPDC1*	In this study
Kluyveromyces marxianus KCTC17555	Source for *KmPDC1*	In this study
Saccharomyces cerevisiae D452-2	Source for *ScPDC1*, *ScPDC5, ScPDC6* *MATα leu2 his3 ura3*	(Nikawa et al., 1991)
BD4	D452-2, *pdc1Δ, pdc5Δ,* evolved 2,3-BD-producing strain	(Kim et al., 2013a)
SOS5	D452-2, *pdc1Δ, pdc5Δ, pdc6Δ*	In this study
SOS5_T2CtPDC1	SOS5, p426TDH3_CtPDC1	In this study
SOS5_T2KmPDC1	SOS5, p426TDH3_KmPDC1	In this study
SOS5_T2ScPDC1	SOS5, p426TDH3_ScPDC1	In this study
SOS5_T2ScPDC5	SOS5, p426TDH3_ScPDC5	In this study
SOS5_T2ScPDC6	SOS5, p426TDH3_ScPDC6	In this study
BD5	SOS5, p423_alsSalsD, p425_BDH1	In this study
BD5_Con	BD5, p426GPD	In this study
BD5_C1CtPDC1	BD5, p406CYC1_CtPDC1	In this study
BD5_G1CtPDC1	BD5, p406GPD2_CtPDC1	In this study
BD5_C2CtPDC1	BD5, p426CYC1_CtPDC1	In this study
BD5_T2CtPDC1	BD5, p426TDH3_CtPDC1	In this study
BD5_Ctnox	BD5, p406GPD2_CtPDC1, pAUR_Llnox	In this study
Plasmids		
pRS406	*URA3*	(Mumberg et al., 1995)

pRS426	*URA3* 2μm origin	(Mumberg et al., 1995)
p406CYC1	pRS406, $CYC1_{prom}$ $CYC1_{term}$	In this study
p406GPD2	pRS406, $GPD2_{prom}$ $CYC1_{term}$	In this study
p426CYC1	pRS426, $CYC1_{prom}$ $CYC1_{term}$	In this study
p426GPD	*URA3* 2μm origin, $TDH3_{prom}$ $CYC1_{term}$	(Mumberg et al., 1995)
p423_alsSalsD	*HIS3* 2μm origin, $TDH3_{prom}$-*alsS*-$CYC1_{term}$ $TDH3_{prom}$-*alsD*-$CYC1_{term}$	(Kim et al., 2014)
p425_BDH1	*LEU2* 2μm origin, $TDH3_{prom}$-*BDH1*-$CYC1_{term}$	(Kim et al., 2013a)
p426TDH3_Llnox	pRS426, $TDH3_{prom}$-*Llnox*-$CYC1_{term}$	In this study
p426TDH3_CtPDC1	pRS426, $TDH3_{prom}$-*CtPDC1*-$CYC1_{term}$	In this study
p426TDH3_KmPDC1	pRS426, $TDH3_{prom}$-*KmPDC1*-$CYC1_{term}$	In this study
p426TDH3_ScPDC1	pRS426, $TDH3_{prom}$-*ScPDC1*-$CYC1_{term}$	In this study
p426TDH3_ScPDC5	pRS426, $TDH3_{prom}$-*ScPDC5*-$CYC1_{term}$	In this study
p426TDH3_ScPDC6	pRS426, $TDH3_{prom}$-*ScPDC6*-$CYC1_{term}$	In this study
p406CYC1_CtPDC1	pRS406, $CYC1_{prom}$-*CtPDC1*-$CYC1_{term}$	In this study
p406GPD2_CtPDC1	pRS406, $GPD2_{prom}$-*CtPDC1*-$CYC1_{term}$	In this study
p426CYC1_CtPDC1	pRS426, $CYC1_{prom}$-*CtPDC1*-$CYC1_{term}$	In this study
p414TEF	*TRP1 CEN6 ARS4*	(Mumberg et al., 1995)
pAUR_Cas9	*AUR1-C CEN6 ARS4* $TEF1_{prom}$-*Cas9*-$CYC1_{term}$	In this study
pAUR_Llnox	*AUR1-C CEN6 ARS4*, $TDH3_{prom}$-*Llnox*-$CYC1_{term}$	In this study

Table 4-2. Primers used in Chapter 4. Bold and capital characters are restriction enzyme sites.

Primers	Restriction site	Sequence
Cloning of *PDC* genes		
F_XmaI_CtPDC1	***Xma*I**	tccc**CCCGGG**aaaatgtctgaaattactttgggtag
R_SalI_CtPDC1	***Sal*I**	acgc**GTCGAC**tttattcttgagcagcgttg
F_XmaI_KmPDC1	***Xma*I**	tccc**CCCGGG**aaaatgtctgaaattactctaggtcg
R_SalI_KmPDC1	***Sal*I**	acgc**GTCGAC**tttattcttgcttggcgtt
F_XmaI_ScPDC1	***Xma*I**	tccc**CCCGGG**atgtctgaaattactttgggtaa
R_SalI_ScPDC1	***Sal*I**	acgc**GTCGAC**ttattgcttagcgttggtag
F_XmaI_ScPDC5	***Xma*I**	tccc**CCCGGG**atgtctgaaataaccttaggtaaata
R_SalI_ScPDC5	***Sal*I**	acgc**GTCGAC**ttattgtttagcgttagtagcg
F_XmaI_ScPDC6	***Xma*I**	tccc**CCCGGG**atgtctgaaattactcttggaaaatac
R_SalI_ScPDC6	***Sal*I**	acgc**GTCGAC**ttattgtttggcatttgtagc
Cloning of *AUR1-C* gene		
F_SnaBI_AUR1-C	***SnaB*I**	agcttgtcacct**TACGTA**aaagtgcccatcagtgttc
R_MfeI_AUR1-C	***Mfe*I**	ataaccgggt**CAATTG**cagaggaaagaataacgcaa

Table 4-3. Kinetic constants of pyruvate decarboxylases. CtPDC1, *C. tropicalis* pyruvate decarboxylase 1; KmPDC1, *K. marxianus* pyruvate decarboxylase 1; ScPDC1, *S. cerevisiae* pyruvate decarboxylase 1; ScPDC5, *S. cerevisiae* pyruvate decarboxylase 5; ScPDC6, *S. cerevisiae* pyruvate decarboxylase 6

Kinetic values	CtPDC1	KmPDC1	ScPDC1	ScPDC5	ScPDC6
K_m (mM)	2.7	7.7	4.7	9.9	8.2
V_{max} (mU/mg protein)	107	383	541	437	415

Table 4-4. Fermentation parameters of wild type and engineered *S. cerevisiae* strains in oxygen-limited batch fermentation with a minimal medium containing 100 g/L glucose.

Parameter	D452-2	BD5_ C1CtPDC1	BD5_ G1CtPDC1	BD5_ C2CtPDC1	BD5_ T2CtPDC1
DCW (g/L)	7.1	1.2	3.4	4.5	6.5
$Y_{glycerol}$ (g/g)	0.023	0.305	0.319	0.280	0.124
$Y_{2,3\text{-BD}}$ (g/g)	0	0.362	0.304	0.256	0.125
$Y_{ethanol}$ (g/g)	0.471	0	0.020	0.085	0.318
$V_{glucose}$ ($g/L \cdot h^{-1}$)	5.32	0.81	2.88	3.29	3.60
$P_{2,3\text{-BD}}$ ($g/L \cdot h^{-1}$)	0	0.29	0.82	0.93	0.49

* 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); $Y_{ethanol}$, ethanol yield (g ethanol/g glucose); $V_{glucose}$, volumetric glucose consumption rate (g glucose/$L \cdot h^{-1}$); $P_{2,3\text{-BD}}$, 2,3-butanediol productivity (g 2,3-butanediol/$L \cdot h^{-1}$).

Table 4-5. Summary of the fed-batch fermentations with 2,3-BD-producing strains.

Parameters	DCW_{Max} (g/L)	Glycerol (g/L)	2,3-BD (g/L)	Ethanol (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD5_CtPDC1	3.9	118.5	121.8	1.2	0.300	0.308	1.52
BD5_CtPDC1_noxE	6.2	32.5	154.3	0.1	0.088	0.404	1.98

1 40
MSEITLGRFFFERLHQLQVDTVFGLPGDFNLALLDKIYEV
41 77
DGMRWAGNANELNAGYAADGYARVNPNGLAALVSTFG
78 117
VGELSLTNAIAGSYSEHVGIINLVGVPSSSAQAKQLLLHH
118 158
TLGNGDFTVFHRMFKNISQTSAFISDPNTAASEIDRCIRDA
159 198
YVYQRPVYIGLPSNLVDVKVPKSLLDKKIDLSLHPNEPES
199 239
QAEVVETVEKFISEASNPVILVDA**C**AIRHNCLKEVAELIAE
240 278
TQFPVFTTPMGKSSVDESNPRFGGVYVGSLSSPDVKEAV
279 316
ESADLVLSVGAMLSDFNTGAFSYNYKTRNVVEFHSDYT
317 355
KIRQATFPGVQMKEALQVLLKTVKKSVNPKYVPAPVPAT
356 395
KAITTPGNNDPVSQEYLWRKVSDWFQEGDVIISETG**T**SAF
396 435
GIVQSKFPKNAIGISQVLWGSIGYATGATCGAAMAAQEID
436 474
PKKRVILFTG**D**G**S**LQLTVQEISTMCKWDCYNTYLYVL**NN**
475 513
D**G**YT**I**ERLIHGEKAQYNDIQPWNNLQLLPLFNAKKYETK
514 553
RISTVGELNDLFTNKEFAVPDRIRMVEIMLPVMDAPANLV
554
AQAKQSAATNAAQE

Figure 4-1. Amino acid sequence of the *C. tropicalis* pyruvate decarboxylase I. Amino acids of Mg^{2+} and ThDP binding loop (bold and underlined) and substrate activation (bold) that are conserved throughout all *PDC*s.

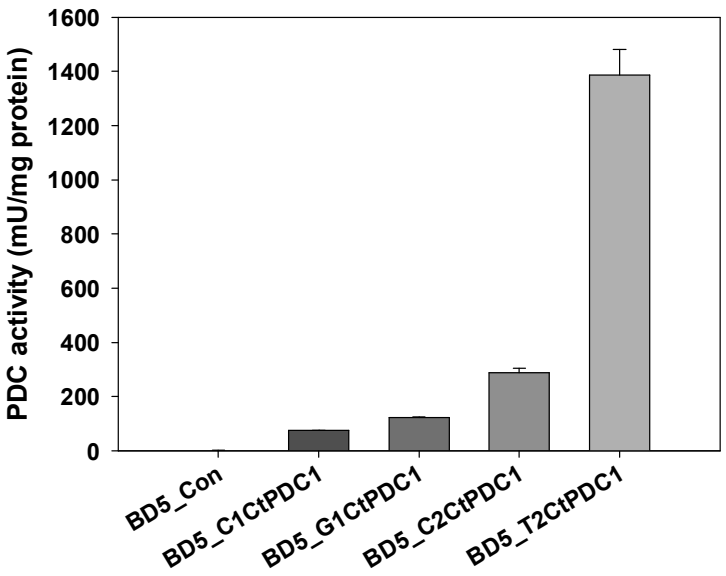
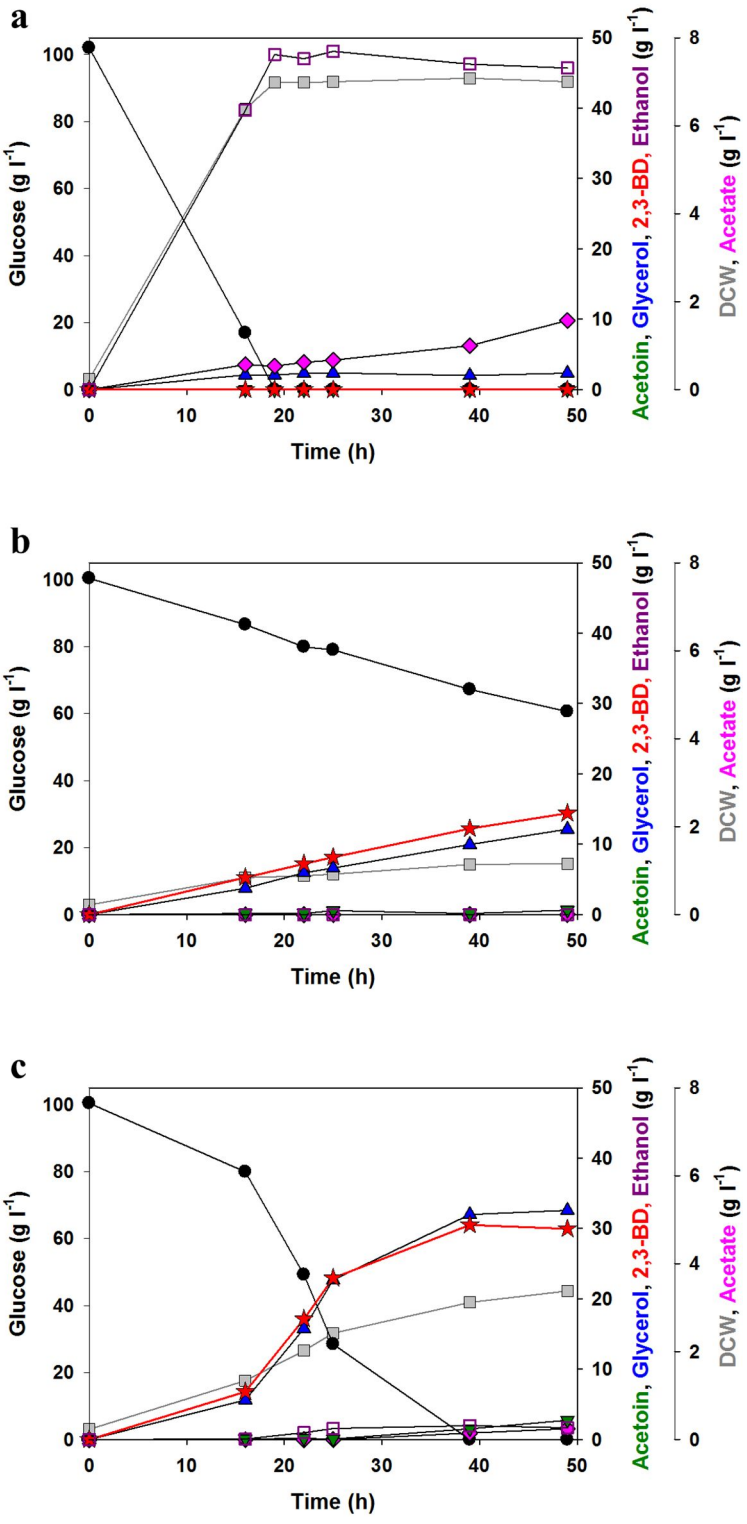


Figure 4-2. *In vitro* pyruvate decarboxylase activity in the engineered strains. One unit of activity was defined as the amount of enzyme oxidizing 1 μmol NADH per minute at the corresponding reaction conditions.


a
b
c
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD, Ethanol (g l-1)
DCW, Acetate (g l-1)
Time (h)
0
10
20
30
40
50
0
20
40
60
80
100
0
10
20
30
40
50
0
2
4
6
8

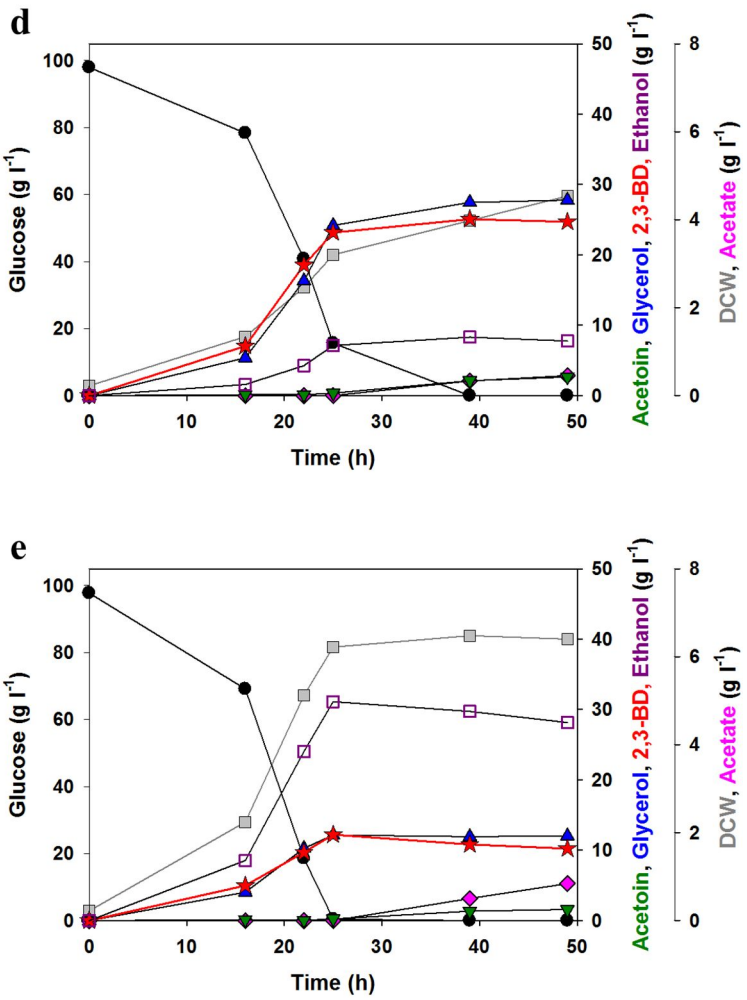
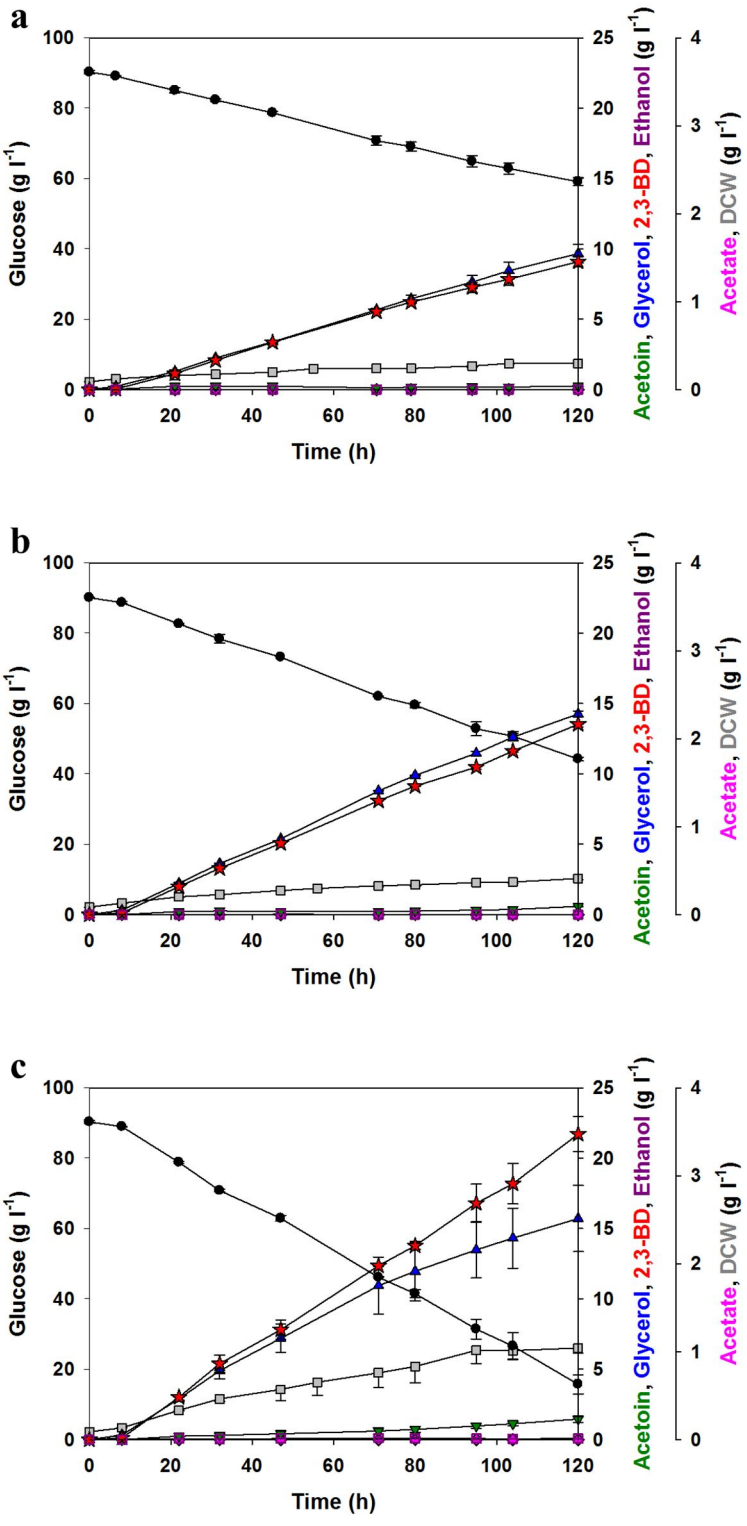


Figure 4-3. Flask cultivation of *PDC* expression strains in YP medium containing 100 g/L glucose. (a) D452-2, (b) BD5_C1CtPDC1, (c) BD5_G1CtPDC1, (d) BD5_C2CtPDC1, and (e) BD5_T2CtPDC1 strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.


a
b
c
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD, Ethanol (g l-1)
Acetate, DCW (g l-1)
Time (h)
0
20
40
60
80
100
120
0
5
10
15
20
25
0
1
2
3
4

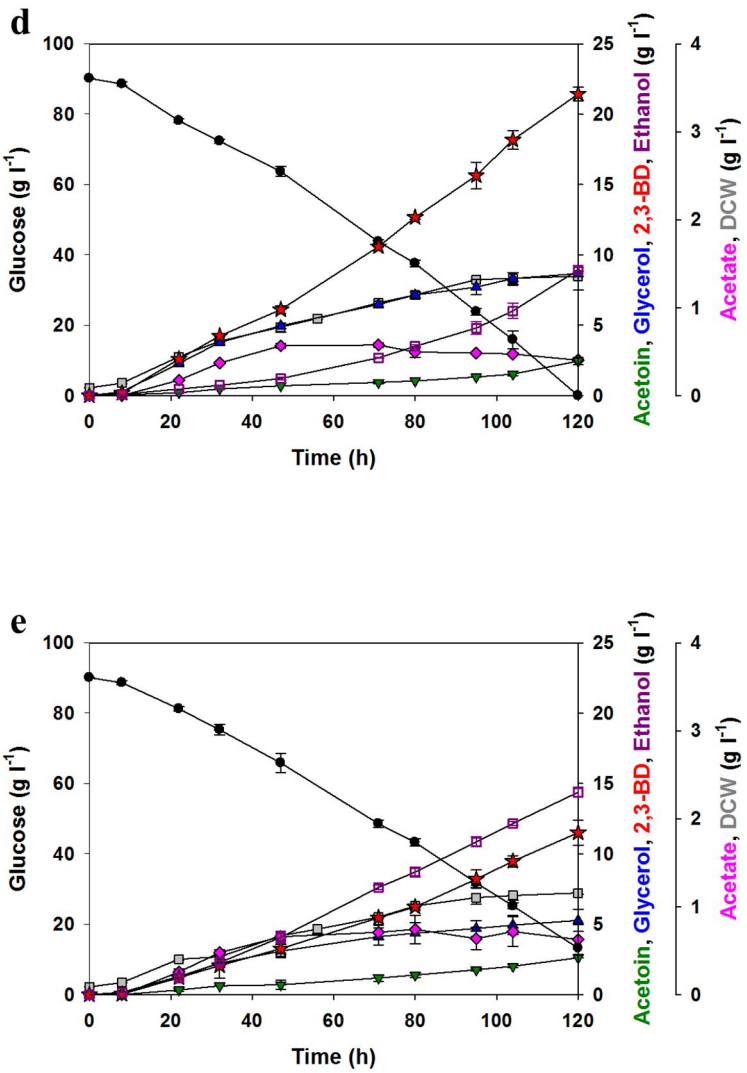


Figure 4-4. Flask cultivation of *PDC* expressing strains in YNB medium containing 90 g/L glucose. (a) BD5_Con, (b) BD5_C1CtPDC1, (c) BD5_G1CtPDC1, (d) BD5_C2CtPDC1, and (e) BD5_T2CtPDC1 strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

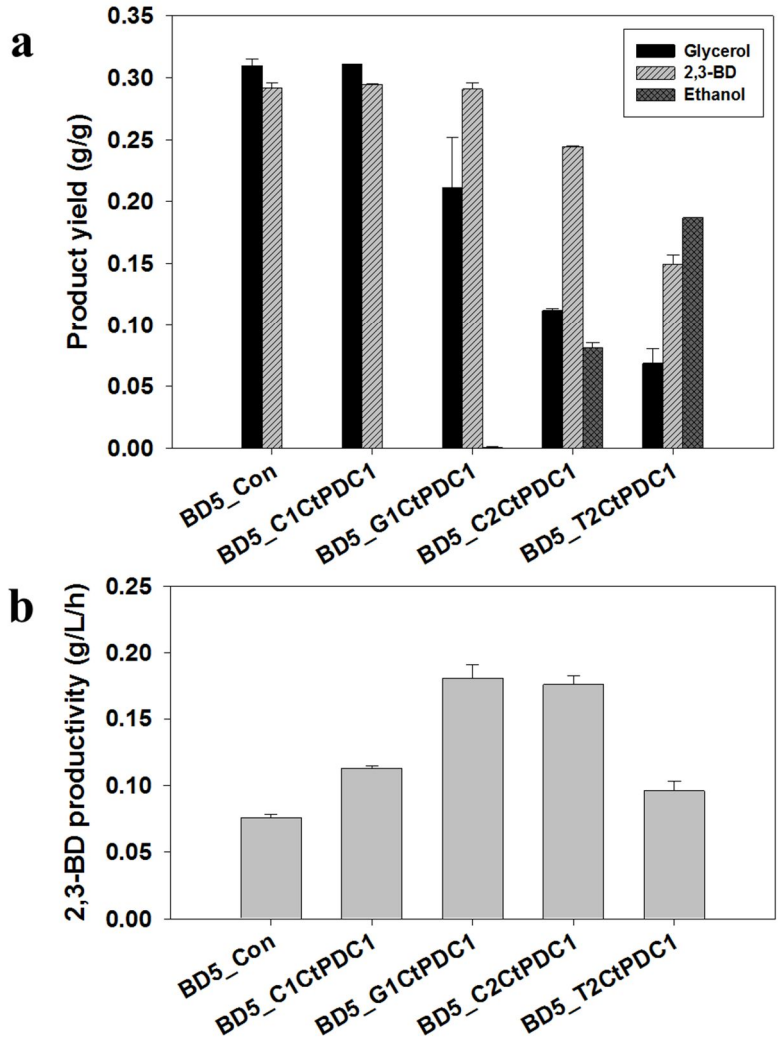
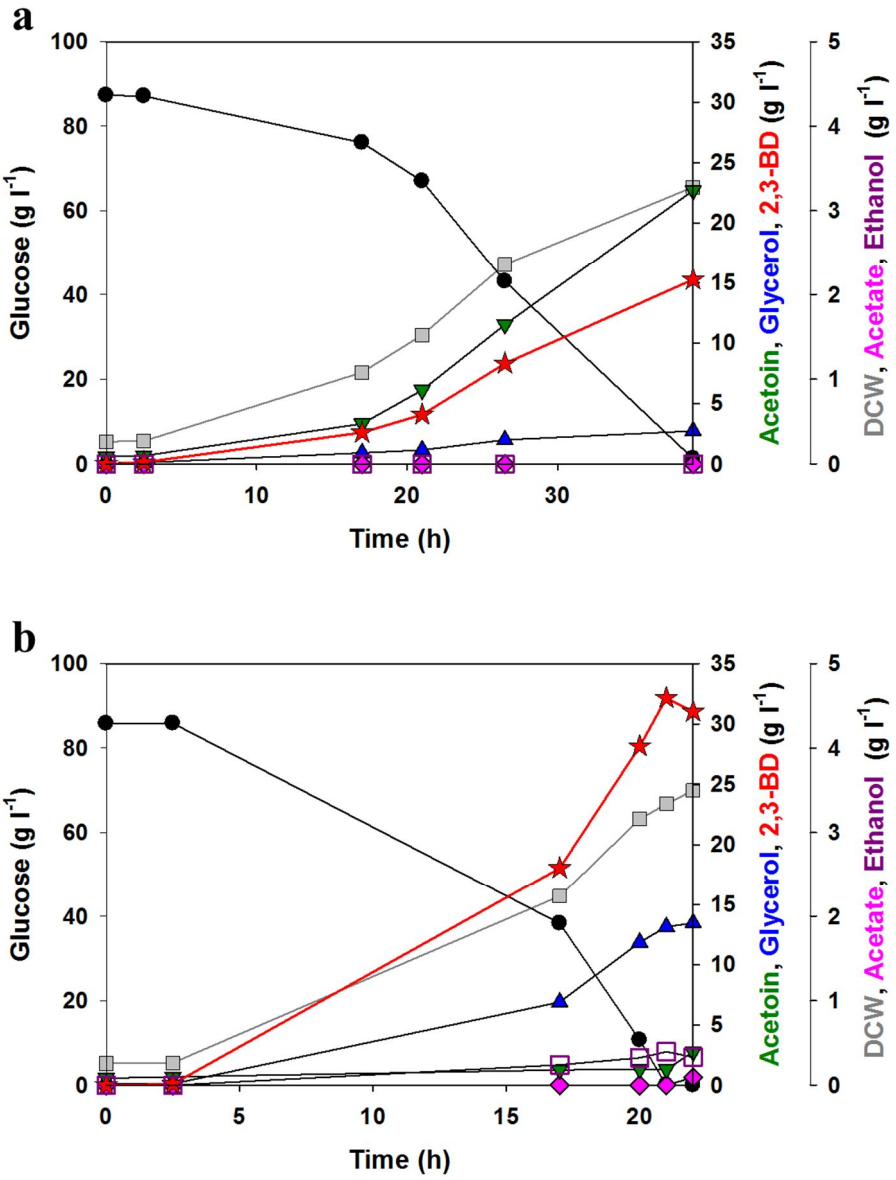


Figure 4-5. Summary of 2,3-BD fermentation with *PDC* expressing strains in YNB containing 90 g/L glucose. (a) Products yield ($g_{Product}/g_{Glucose}$), (b) 2,3-BD productivity ($g_{2,3\text{-}BD}$/L/h).


a
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
DCW, Acetate, Ethanol (g l-1)
Time (h)
b
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
DCW, Acetate, Ethanol (g l-1)
Time (h)

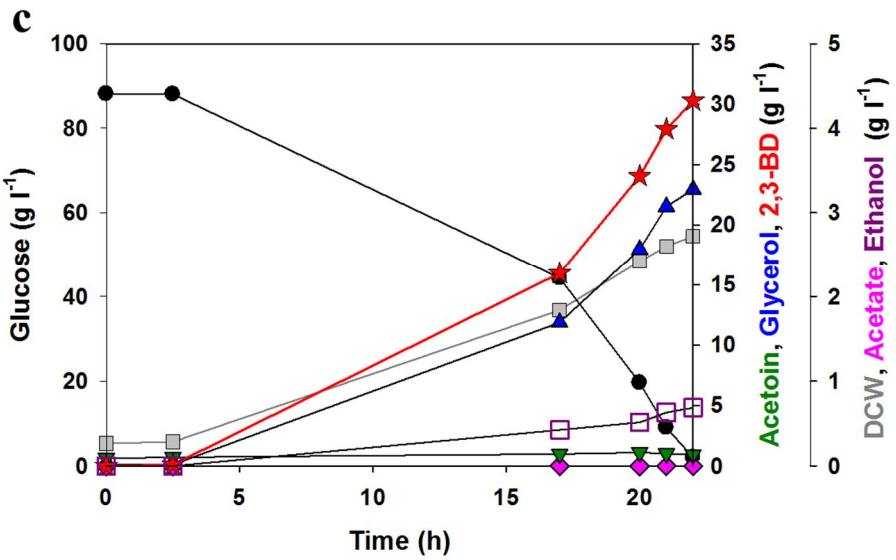


Figure 4-6. Batch fermentation of BD5_Ctnox strain with different oxygen supply. (a) fully-aerobic condition (100% air), (b) oxygen-limited condition (50% air), (c) oxygen-limited condition (25% air). Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

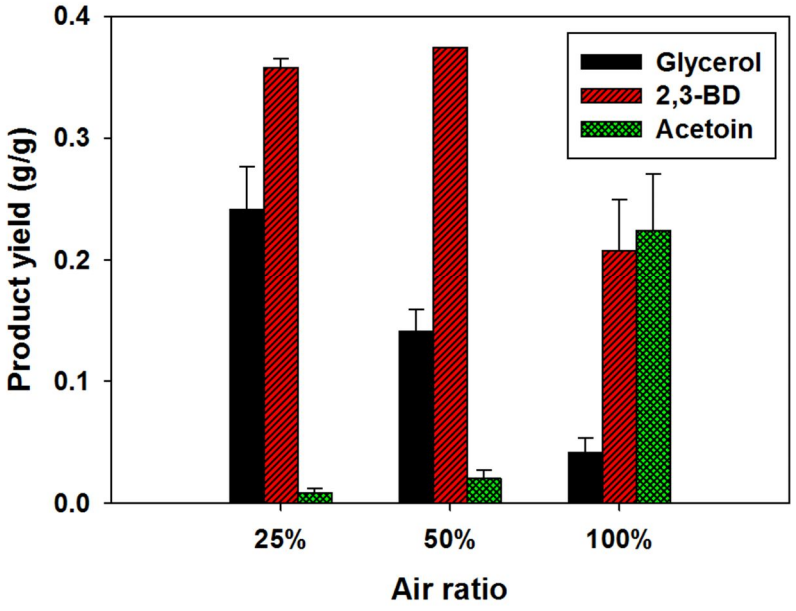
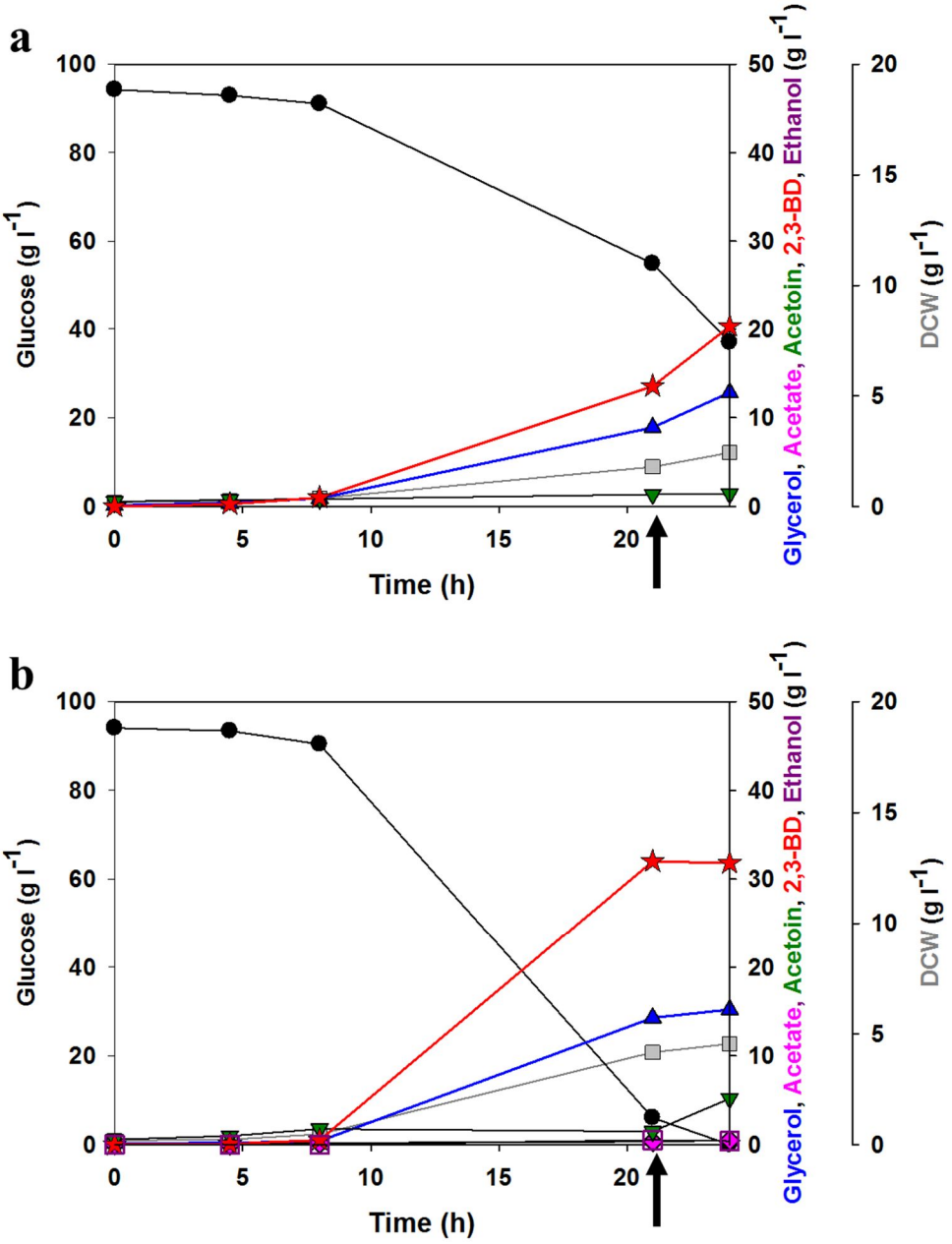


Figure 4-7. Product yields of the BD5_Ctnox strain in different oxygen levels


a
b
Glucose (g l-1)
Glycerol, Acetate, Acetoin, 2,3-BD, Ethanol (g l-1)
DCW (g l-1)
Time (h)
0
5
10
15
20
0
20
40
60
80
100
0
10
20
30
40
50
0
5
10
15
20

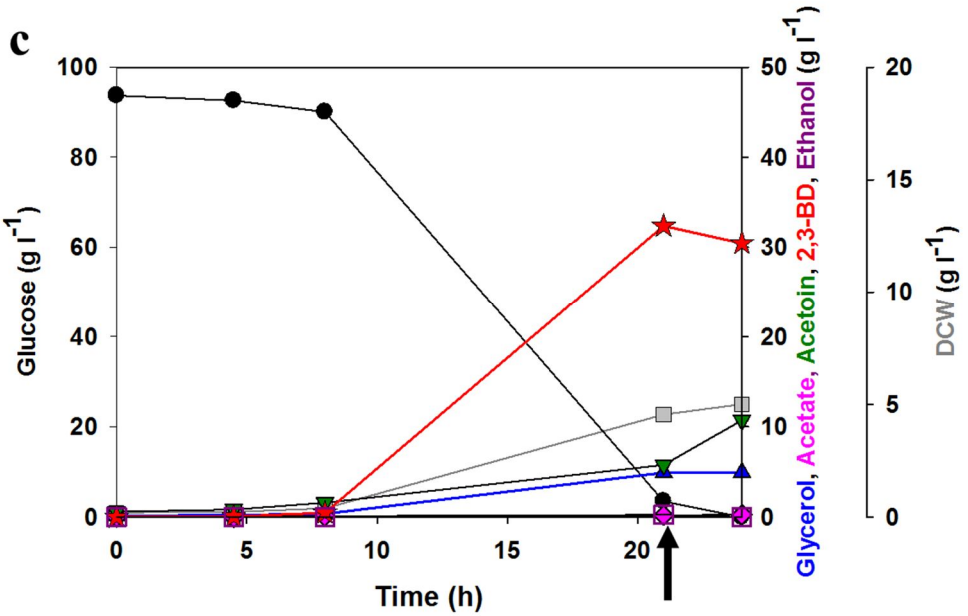


Figure 4-8. Batch fermentation of (a) BD5_G1CtPDC1 strain in aerobic condition (100% air), (b) BD5_Ctnox strain in microaerobic condition (50% air), and (c) BD5_Ctnox strain in aerobic condition (100% air). Arrows indicate the sample preparation time for metabolites analysis. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

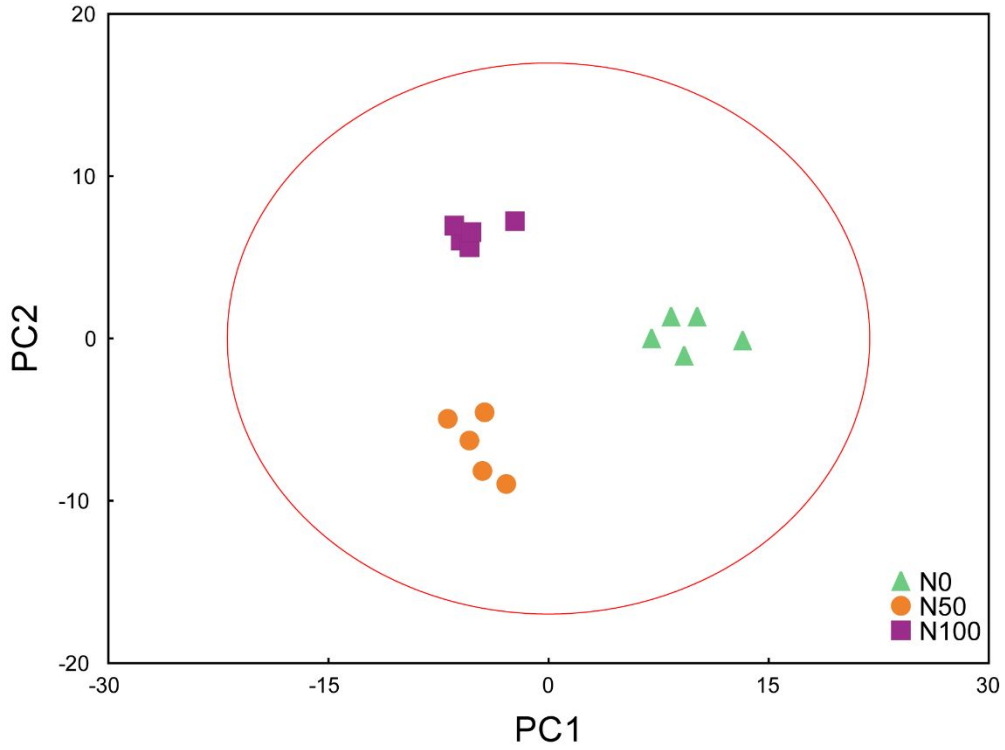


Figure 4-9. Principal component analysis (PCA) of intracellular metabolite profiles of BD5_G1CtPDC1 and BD5_Ctnox in different aerobic conditions.

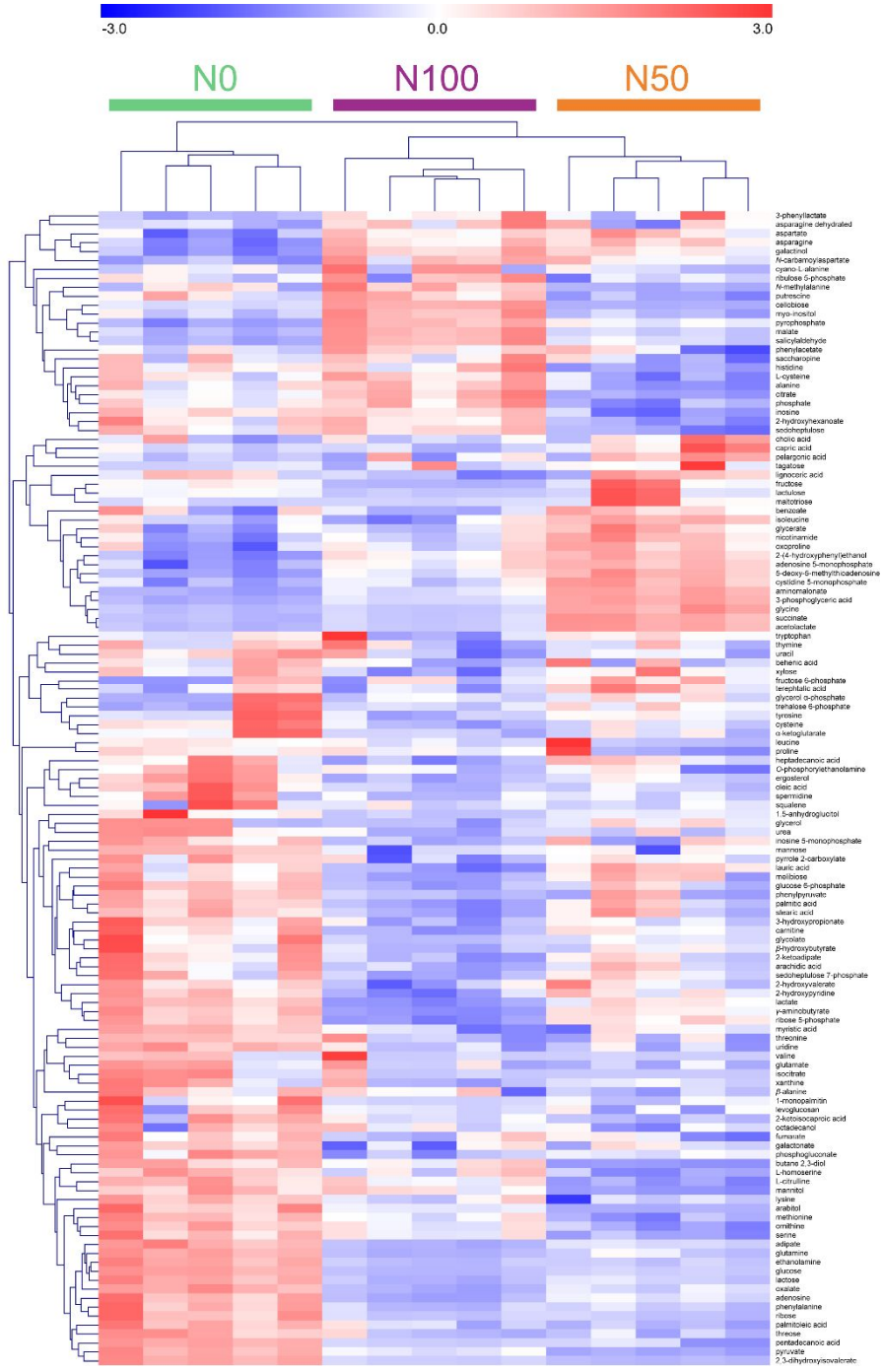


Figure 4-10. Hierarchical clustering analysis of metabolite profiles of BD5_G1CtPDC1 and BD5_Ctnox in different aerobic conditions.

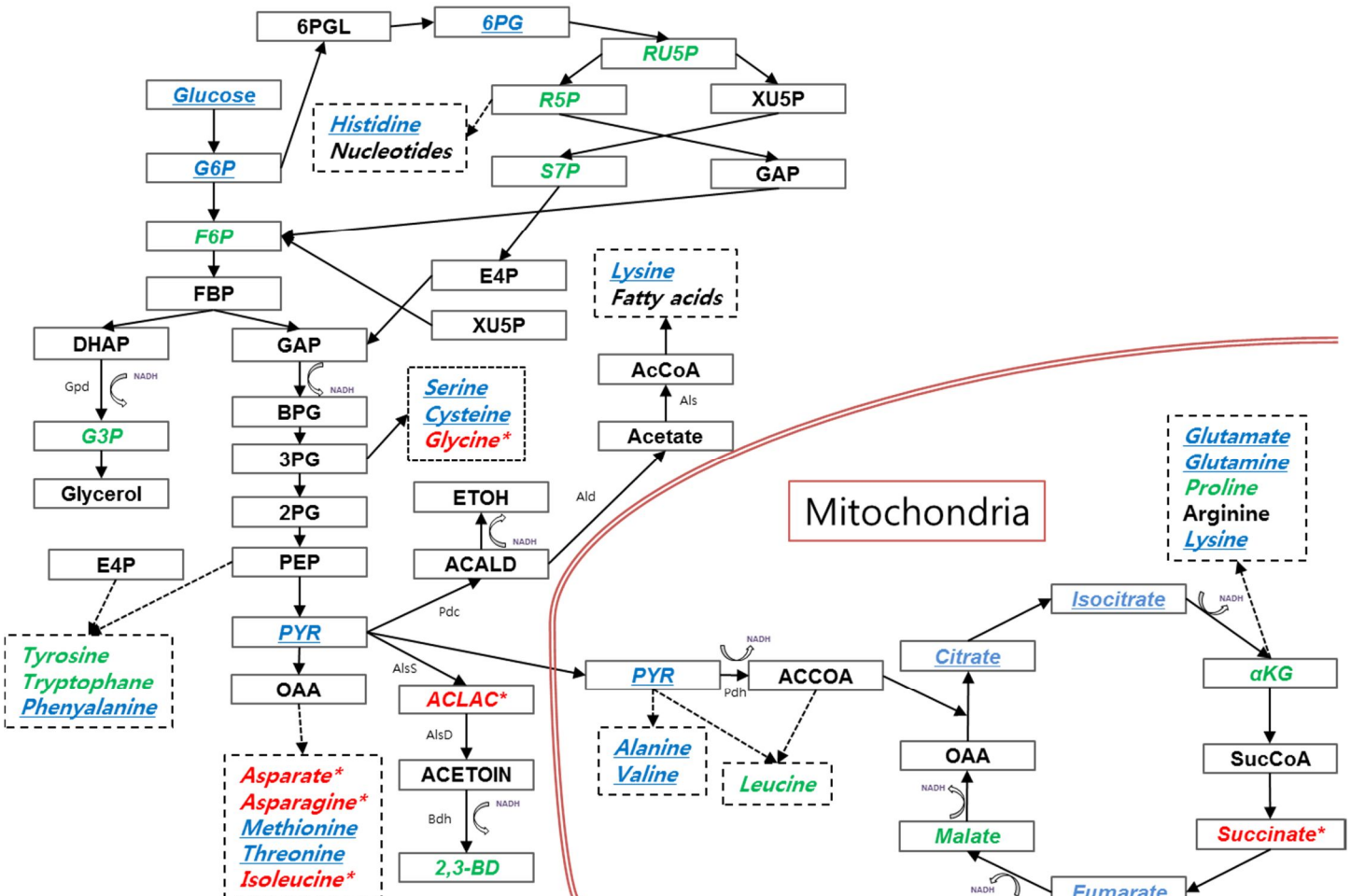

6PGL
6PG
RU5P
Glucose
R5P
XU5P
Histidine
Nucleotides
G6P
S7P
GAP
F6P
E4P
Lysine
Fatty acids
FBP
XU5P
DHAP
GAP
AcCoA
Gpd
NADH
NADH
Als
BPG
Serine
Cysteine
Glycine*
G3P
3PG
Acetate
Glycerol
2PG
ETOH
Ald
Mitochondria
Glutamate
Glutamine
Proline
Arginine
Lysine
E4P
PEP
ACALD
NADH
Isocitrate
NADH
Pdc
PYR
Citrate
αKG
Tyrosine
Tryptophane
Phenyalanine
NADH
PYR
ACCOA
OAA
AlsS
ACLAC*
Pdh
Alanine
Valine
Leucine
OAA
SucCoA
AlsD
ACETOIN
NADH
Malate
Succinate*
Bdh
NADH
2,3-BD
Asparate*
Asparagine*
Methionine
Threonine
Isoleucine*
NADH
Fumarate

Figure 4-11. Comparison of intracellular metabolites between BD5_G1CtPDC1 at aerobic condition (N0) and BD5_Ctnox at microaerobic condition (N50). Compounds with italic letters indicates identified and quantified metabolites. The underlined characters are up-regulated metabolites in N0 condition, and the metabolites with asterisk (*) are up-regulated metabolites in N50 condition. The other metabolites without underlined character and asterisk are not statistically significant ($p > 0.05$). G6P; glucose-6-phosphate, F6P; fructose-6-phosphate, FBP; fructose-1,6-bisphosphate, DHAP; dihydroxyacetonephosphate, G3P; glycerol-3-phosphate; GAP; glycealdehyde-3-phosphate, BPG; 1,3-bisphosphoglycerate, 3PG; 3-phosphoglycerate, 2PG; 2-phosphoglycerate, PEP; phosphoenolpyruvate, PYR; pyruvate, OAA; oxaloacetic acid, 6PGL; 6-phosphogluconolactone, 6PG; 6-phosphogluconate, RU5P; ribulose-3-phosphate, R5P; ribose-5-phosphate, XU5P; xylulose-5-phosphate, S7P; sedoheptulose-7-phosphate; E4P; erythrose-4-phosphate, ACALD; acetaldehyde, ETOH; ethanol, AcCoA; acetyl-CoA, ACLAC; acetolactate, 2,3-BD; 2,3-butanediol, αKG; α-ketoglutarate, SucCoA; succinyl-CoA.

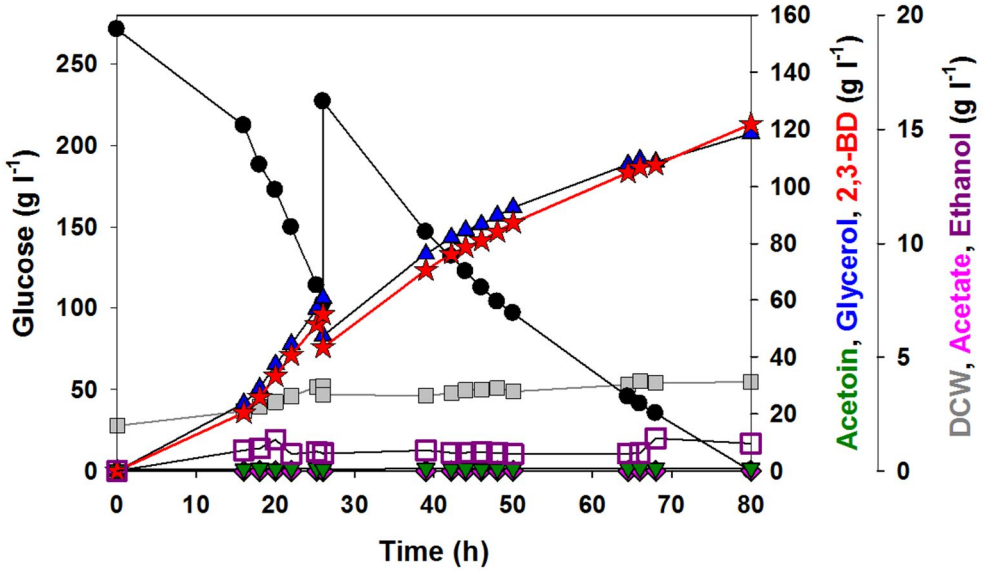


Figure 4-12. Fed-batch fermentation with the BD5_Ct strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

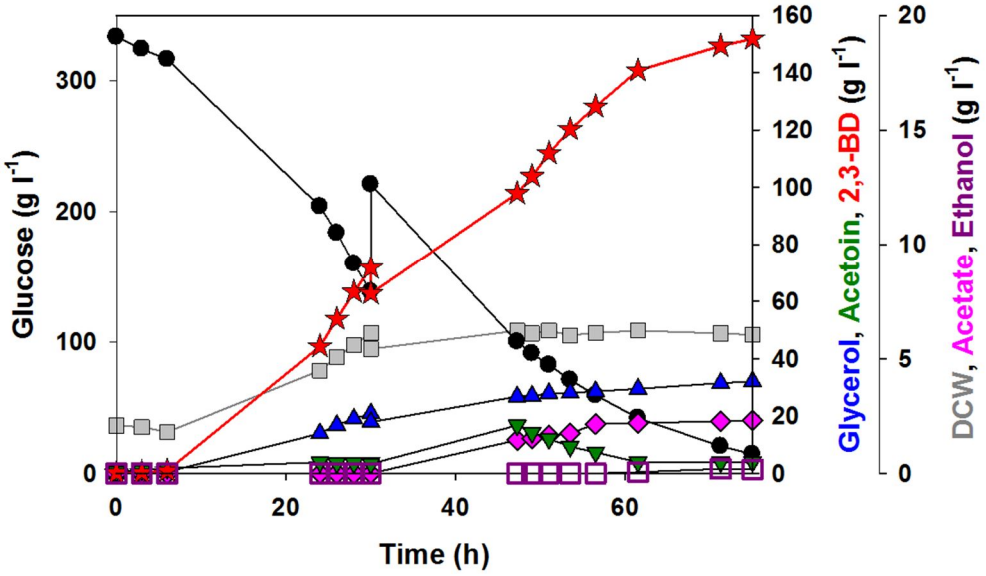


Figure 4-13. Fed-batch fermentation with the BD5_Ctnox strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

Chapter 5.

Improved 2,3-butanediol yield by double deletion of glycerol-3-phosphate dehydrogenase in Pdc-deficient *S. cerevisiae*

5.1. Summary

In this chapter, the endogenous glycerol-3-phosphate dehydrogenase genes (*GPD1* and *GPD2*) were deleted in the BD5_T2nox strain by the Cas9-CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system. Through the deletion of *GPD1* or *GPD2* genes, the engineered strain produced higher yield of 2,3-BD and lower yield of glycerol than those of the control strain. Additionally, glycerol production could be completely blocked following the mutation of both two *GPD* genes, and thereby the 2,3-BD production yield increased. Although deletion of both *GPD* genes could block glycerol production, the glucose consumption, cell growth, and 2,3-BD productivity were significantly reduced in batch fermentations at high concentrations of glucose. Since glycerol is a compatible solute for osmoregulation in *S. cerevisiae* as well as a redox sink, fed-batch fermentation with low concentration of glucose was carried out to increase cell growth and 2,3-BD productivity. As a result, the BD5_Ctnox_dGPD1dGPD2 strain could produce 108.6 g/L of 2,3-BD in 70 h cultivation. 2,3-BD yield was 0.462 $g_{2,3\text{-}BD}/g_{Glucose}$ (92.4% of theoretical yield) which is 11.6% higher than the results from the fed-batch fermentation with the BD5_T2nox strain (80.8%). These results demonstrate that the carbon flux could be redirected from glycerol to 2,3-BD efficiently through expression of NADH oxidase and deletion of the *GPD1* and *GPD2* genes.

5.2. Introduction

2,3-BD could be efficiently produced by the engineered Pdc-deficient *S. cerevisiae* strains harboring the bacterial 2,3-BD biosynthetic pathway. However, the 2,3-BD-producing Pdc-deficient *S. cerevisiae* strains produced a large amount of glycerol as a byproduct because cofactor imbalance in cytosol. In the 2,3-BD-producing Pdc-deficient strains, one mole of NADH was additionally produced per one mole of 2,3-BD because two moles of pyruvate were converted into one mole of 2,3-BD with one mole of NADH oxidized. Thus, in the previous study, cofactor engineering of the 2,3-BD-producing Pdc-deficient strain by expression of NADH oxidase reduced glycerol yield and increased 2,3-BD yield. However, in spite of the optimized expression levels of NADH and fermentation conditions, over 30 g/L of glycerol was produced as a byproduct (Kim et al., 2015). Glycerol production in the 2,3-BD-producing strains has several problems with industrial applications. 2,3-BD yield was low compared to bacterial production systems because still a large part of a carbon source was used for glycerol production. In addition, high concentration of glycerol increases separation cost because glycerol co-exists with 2,3-BD even after a distillation process from a fermentation broth and additional separation steps are needed (Xiu & Zeng, 2008).

Glycerol production in *S. cerevisiae* is catalyzed by NADH-dependent glycerol-3-phosphate dehydrogenase encoded by the *GPD* gene and glycerol-3-phosphate phosphatase encoded by the *GPP* gene. *S. cerevisiae* is known to possess two *GPD* isozymes, *GPD1* and *GPD2*. It was reported that deletion of the *GPD1* or *GPD2* gene decreased glycerol yield (Guo et al., 2009; Kim et al., 2012; Michnick et al., 1997a; Nissen et al., 2000a; Valadi et al., 1998b), and deletion of both of *GPD* genes could completely block glycerol formation (Hubmann et al., 2011; Medina et al., 2010) . In this chapter, both *GPD1* and *GPD2* genes were deleted for blocking glycerol formation as a byproduct. Glycerol acts as a redox sink for balancing the ratio of NADH and NAD^+ in the cytoplasm through the formation of glycerol at the cost of one mol NADH per one mole glycerol produced (Albers et al., 1996; Nissen et al., 2000a). The respiratory chain and cofactor shuttle between cytosol and mitochondria could oxidize cytosolic NADH, but were not enough to oxidize excess cytosolic NADH (Bakker et al., 2001; van Dijken & Scheffers, 1986). Thus, the additional NADH formed by blocking glycerol production in the 2,3-BD-producing strains should be oxidized to NAD^+ through alternative pathways for maintaining cellular metabolism (Vemuri et al., 2007). Therefore, in this chapter, oxidation reaction of excess NADH by glycerol production was substituted by the reaction of the NADH oxidase. The cofactor imbalance of the engineered

2,3-BD strains with the blocked glycerol production relieved by expression of NADH oxidase. Thus, the 2,3-BD-producing strain with the deleted *GPD* genes could be constructed by co-expression of NADH oxidase.

5.3. Materials and Methods

5.3.1. Construction of plasmids

Plasmids used in this chapter are summarized in Table 5-1. The primers used for construction of gBlock and repair DNA are listed in Table 5-2. *E. coli* TOP10 (Invitrogen, Carlsbad, CA) was used for gene cloning and manipulation. *E. coli* transformants were grown in Lysogeny Broth (LB) medium with 100 μg/mL of ampicillin. To construct Cas9 expression plasmids, ORF of *AUR1-C* genes were amplified by PCR from pAUR101 plasmids, using the primers in Table 5-2. The amplified DNA fragments were ligated into appropriate restriction sites in p414_TEF1p_Cas9_CYC1t plasmids. For construction of guideRNA expression plasmids for *GPD1* and *GPD2* deletion, guideRNA expression cassette were constructed by overlap-PCR method as shown in Figure 5-1. First, fused DNA fragments of promoter and targeting sequence DNA for *GPD1* or *GPD2* were amplified by F1 and R1. Second, fused DNA fragments of targeting sequence DNA for *GPD1* or *GPD2*, guideRNA and terminator were amplified by F2 and R2 primer. Then, using F1 and R2 primer, guide expression cassettes were constructed. The DNA fragments were ligated into restriction site of *Sac*I and *Kpn*I in pRS42H-gLEU2.

5.3.2. Yeast transformation and construction of recombinant *S. cerevisiae* strains

Transformation of plasmids for deleting *GPD1* and *GPD2* genes was performed using a spheroplast transformation kit (BIO 101, Vista, CA). To select transformants, *S. cerevisiae* strains were routinely cultivated aerobically at 30^{o}C in YNB medium (6.7 g/L yeast nitrogen base and appropriate nucleotides and amino acid). 20 g/L glucose and 1 g/L ethanol was used as carbon sources. For construction of *GPD1* and *GPD2* deletion strains, the BD5_T2nox_Cas9 strain was constructed by transformation of pAUR_Cas9 plasmids into BD5_T2nox strain. Then, the pRS42H-gGPD1 or pRS42H-gGPD2 plasmids were transformed into the BD5_T2nox_Cas9 strain with repair DNA fragments amplified by PCR with F3 and R3 primers in Table 5-2. For selection of transformants, YNB medium with 0.1 μg/mL of aureobasidin A or 250 ug/mL of hygromycin B was used. For construction of the BD5_dGPD1 and BD5_dGPD2 strains, the BD5_T2nox_dGPD1 and BD5_T2nox_dGPD2 strains were serial cultivated in YNB medium containing uracil. The Cas9 and guideRNA expression plasmids were cured after deletion of *GPD1* and *GPD2* by serial cultivation on YNB medium without aureobasidin A and hygromycin B. For construction of additional CtPDC1 expression strain, the BD5_Ctnox_dGPD1dGPD2 strain was constructed by transformation of pRS42H-gADH1 plasmid and repair DNA

from PCR reaction with F3_Repair_ADH1_TDH3p and R3_Repair_ADH1_CYC1t as primers. The resulting recombinant *S. cerevisiae* strains are listed in Table 5-1.

5.3.3. 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 microaerobic conditions in 250 mL flasks at 80 rpm. In order to prepare inoculums, engineered *S. cerevisiae* cells were cultivated during for 120 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 48 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 100 g/L of glucose, 0.7 g/L of ethanol and 50 mM potassium phthalate at pH 5.5 adjusted by NaOH.

Batch fermentations with bioreactor were performed in 500 mL YP medium containing 300 g/L glucose using 1 L-bench-top fermentor (Fermentec, Korea) at 30°C. The medium pH was maintained at 5.5 with intermittent addition of 5N NaOH and dissolved oxygen (DO) levels were

monitored with O_2 sensor (Mettler Toledo, Switzerland). The culture medium was agitated at 200-400 rpm and aerated with air flow rate of 1-2 vvm according to DO levels.

Fed-batch fermentations with bioreactor were carried out in 500 ml YP medium containing initial 100 g/L glucose using 1 L-bench-top fermentor (Fermentec, Korea) at 30^{o}C. The medium pH was maintained at 5.5 with 5N NaOH solution and dissolved oxygen (DO) levels were monitored with O_2 sensor (Mettler Toledo, Switzerland). The culture medium was agitated at 300-500 rpm and aerated with air flow rate of 1-2 vvm according to DO levels in medium. During the fed-batch fermentation, DO levels were kept under 2.0. The grown cells prepared from flask culture were inoculated at the initial concentration of 2.0 g DCW/L. At the time glucose was totally consumed, 150 g/L of glucose was dumped into the medium from 800 g/L of glucose stock solution.

5.3.4. 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 $g_{Drycell}/L/OD_{600}$. Glucose, glycerol, acetoin, 2,3-BD, and ethanol were analyzed by a high-performance liquid chromatography (1100 series, Agilent, CO) equipped

with a Rezex ROA-organic acid column (Phenomenex, CA). Metabolites were detected by a refractive index (RI) detector.

5.4. Results

5.4.1. Construction of *GPD* deletion strains by Cas9-CRISPR system

Recently, the bacterial type II Cas-CRISPR system gained attention as a powerful genome-editing tool (Zhang et al., 2014). The Cas-CRISPR system has been used with great success in various microorganisms (Cong et al., 2013; DiCarlo et al., 2013; Mali et al., 2013a; Mali et al., 2013b; Shalem et al., 2014). Cas9 functions as an RNA-guided endonuclease, and generates double-strand break (DSB) followed by the protospacer adjacent motif (PAM) at the guide RNA targeting sequence (Zhang et al., 2014). As shown in Figure 5-2, the *GPD1* and *GPD2* genes were deleted by the Cas9-CRISPR system. In the middle of ORFs of the *GPD1* and *GPD2* genes, termination codons (TAA) were generated by point mutation. Therefore, the *GPD1* and *GPD2* genes were not expressed in the BD5_T2nox_dGPD1, BD5_T2nox_dGPD2, and BD5_T2nox_dGPD1dGPD2 strain. Then, BD5_dGPD1 and BD5_dGPD2 strains were constructed by curing *noxE* expression plasmids (p426TDH_Llnox) (Table 5-3). After serial cultivations in non-selective medium, *noxE* plasmids were cured for 31.3% and 37.5% of total tested colonies from the BD5_T2nox_dGPD1 and BD5_T2nox_dGPD2 respectively. In the other hand, the *noxE* expression plasmids could not be cured from the BD5_T2nox_dGPD1dGPD2 strain even though 120 colonies were tested.

5.4.2. Flask cultivation of *GPD* deletion strains

In Figure 5-3 and Table 5-4, maximum cell mass of the BD5_T2nox strain (1.3 g_{DCW}/L) was reduced by the deletion of the *GPD* genes. In the previous work, single deletion of the *GPD1* or *GPD2* gene in *S. cerevisiae* gave a similar (Valadi et al., 1998a) or slightly decreased specific growth rate compared with the wild type (Nissen et al., 2000a). Deletion of the *GPD* genes also affected consumption of glucose, production of glycerol, acetoin, and 2,3-BD. In an aspect of glycerol production, individual deletion of *GPD1* and *GPD2* decreased glycerol yield to 0.086 and 0.083 $g_{Glycerol}/g_{Glucose}$ respectively from 0.166 $g_{Glycerol}/g_{Glucose}$ for the control strain. Meanwhile, double deletion of both *GPD1* and *GPD2* resulted in no production of glycerol. Sole deletion of the *GPD* genes and double deletion of *GPD1* and *GPD2* reduced glucose consumption rate from 1.13 g/L/h (the BD5_T2nox strain) to 0.62 g/L/h (the BD5_T2nox_dGPD1 strain), 0.64 g/L/h (the BD5_T2nox_dGPD2 strain), and 0.38 g/L/h (the BD5_T2nox_dGPD1dGPD2 strain). Reduced cell mass by deletion of the NADH-dependent *GPD* genes decreased glucose consumption rate as reported elsewhere (Nissen et al., 2000a). Along with a decrease in glucose consumption rate, 2,3-BD production rates were reduced by the *GPD* deletion. Sole deletion of *GPD1* or *GPD2*, and the double deletion gave the 2,3-BD productivities of 0.19, 0.20, and 0.14 g/L/h, which were 58%, 61%,

and 42% of the corresponding value for the BD5_T2nox strain, respectively. However, the double deletion of *GPD1* and *GPD2* gave a high 2,3-BD yield (0.363 $g_{2,3\text{-}BD}/g_{Glucose}$) to the BD5_T2nox strain (0.333 $g_{2,3\text{-}BD}/g_{Glucose}$). It is clear that deletion of the *GPD1* and *GPD2* genes exerted an effect on the reduced production of glycerol and increased ethanol yield compared with the control strain.

5.4.3. Batch and fed-batch fermentation with *GPD* deletion strain

To test the 2,3-BD production performance of the BD5_Ctnox_dGPD1dGPD2 strain, the batch fermentation experiments were performed with glucose as a sole carbon source (Figure 5-4 and Table 5-5). In batch fermentation, total 246.8 g/L of glucose was consumed during 184 h cultivation from initial 300 g/L of glucose. Cell growth was retarded and initial 3.4 g_{DCW}/L of cell mass was not increased until the end of fermentation. Finally, 99.4 g/L of 2,3-BD was produced with productivity of 0.62 $g_{2,3\text{-}BD}$/L/h. The produced ethanol and glycerol was negligible (0.2 g/L and 0.4 g/L, respectively), but a small amount of acetate (1.4 g/L) was produced. From the middle of fermentation, acetoin accumulated and final acetoin concentration was 11.3 g/L. 2,3-BD yield was 0.418 $g_{2,3\text{-}BD}/g_{Glucose}$ which is 83.6% of theoretical yield of 2,3-BD.

The fed-batch fermentation was carried out to evaluate whether the BD5_Ctnox_dGPD1dGPD2 strain could be a promising host strain for producing 2,3-BD. Aeration conditions were controlled with two stages for maximizing 2,3-BD by minimizing by-products formation according to the results of the batch fermentations of the NADH oxidase strains. In the first stage (< 40 h), fermentation medium was fully aerated for maximizing *in vivo* NADH oxidase activity. As a result, 7.3 g/L of acetoin was produced as a byproduct with 55.13 g/L of 2,3-BD at 39 h cultivation. In the second stage, the fermentation medium was under oxygen-limited condition, the produced acetoin could be reduced to 2,3-BD. Maximum DCW was 5.3 g_{DCW}/L increased from initial 3.4 g_{DCW}/L. The production of glycerol and ethanol was blocked, and only a trace amount of acetoin was produced as a byproduct (0.1 g/L). As a result, final 108.6 g/L of 2,3-BD was produced with 1.55 $g_{2,3\text{-}BD}$/L/h of productivity. The overall yield of 2,3-BD was 0.462 $g_{2,3\text{-}BD}/g_{Glucose}$ which is 92.4% of theoretical yield. The 2,3-BD concentration, yield, and productivity were improved in the fed-batch fermentation compared with the batch fermentation with the BD5_Ctnox_dGPD1dGPD2 strain.

5.5. Discussion

Glycerol which is a main fermentative byproduct of *S. cerevisiae* was overproduced in the engineered 2,3-BD-producing *S. cerevisiae*. The overproduction of glycerol could block an economical production of 2,3-BD by reducing 2,3-BD yield and increasing separation cost (Kim et al., 2015; Xiu & Zeng, 2008). In this chapter, the glycerol-3-phosphate dehydrogenase (Gpd)-deficient strains were constructed by removing glycerol biosynthetic pathway. From the fed-batch fermentation with the BD5_Ctnox_dGPD1dGPD2 strain, maximum 106.8 g/L of 2,3-BD was produced with overall yield of 0.462 $g_{2,3\text{-BD}}/g_{Glucose}$ which corresponds to 92.4% of theoretical yield of 2,3-BD. The yield is not only the highest value among the reported 2,3-BD production with *S. cerevisiae*, but is comparable to other bacterial systems.

Glycerol is formed in order to balance the ratio of NADH and NAD^+ levels in the cytoplasm (Albers et al., 1996). The excess NADH produced by the assimilation of sugars should be recycled to NAD^+ at a substrate level because of the limited respiratory chain and cofactor shuttle activities (Rigoulet et al., 2004; van Dijken & Scheffers, 1986). Especially, glycerol was overproduced in Pdc-deficient 2,3-BD-producing *S. cerevisiae* because NADH accumulated in cytosol by cofactor imbalance (Kim et al., 2015; Kim et al., 2013a; Kim et al., 2014). As reported previously (Hubmann et al.,

2011; Michnick et al., 1997a; Valadi et al., 1998a), reduction and elimination of glycerol production were achieved by the deletion of *GPD1* and/or *GPD2* genes in Pdc-deficient 2,3-BD-producing *S. cerevisiae*. Through the deletion of the *GPD* genes, 2,3-BD yield was significantly improved by changing carbon flux from glycerol to 2,3-BD concomitant with reduced maximum DCW and glucose consumption rate. The reduced cell growth rate and glucose consumption rate in the *gpdΔ S. cerevisiae* strains were a common phenomenon as reported previously (Björkqvist et al., 1997; Guo et al., 2011; Kim et al., 2012; Medina et al., 2010; Michnick et al., 1997b; Nissen et al., 2000b). Especially, double deletion mutant of *gpd1* and *gpd2* exhibited an inability to grow anaerobically because of accumulation of NADH in cytosol unless other electron acceptor was available (Medina et al., 2010). In this study, the *gpd* null mutant strains was constructed when NADH oxidase was co-expressed. In these strains, the accumulated cytosolic NADH by impaired glycerol synthesis might be oxidized to NAD^+ by NADH oxidase using molecular oxygen as an electron acceptor. The growth defect induced by an accumulation of cytosolic NADH could be more critical in the Pdc-deficient *S. cerevisiae* harboring 2,3-BD biosynthetic enzymes than wild type *S. cerevisiae* strains because biosynthesis of 2,3-BD is an NADH-generating pathway as mentioned above. Thus, the expression of NADH oxidase seems to be indispensable to grow the engineered *S. cerevisiae* on glucose.

In batch fermentation with high concentration (300 g/L) of glucose, cells could not grow in fermentation medium (Figure 5-4). The plausible reason for the retarded growth on high concentration of glucose is impaired osmotolerance in the Gpd-deficient strain. Glycerol produced by *S. cerevisiae* acts as not only relieving a cytosolic NADH level, but also an intracellular solute for osmoregulation (Wang et al., 2001). Glycerol is a main compatible solute in *S. cerevisiae*, and intracellularly accumulated when cells are exposed to low extracellular water activity (Nevoigt & Stahl, 1997). Thus, when *S. cerevisiae* is exposed to highly osmolar media, cells respond by rapid intracellular accumulation of glycerol to counteract their dehydration. However, the BD5_Ctnox_dGPD1dGPD2 strain which both of the *GPD* genes were deleted, could not grow on medium with 300 g/L of glucose because of high osmolality (Figure 5-4). As a result, the BD5_Ctnox_dGPD1dGPD2 strain produced 2,3-BD with low productivity. On the other hand, the BD5_Ctnox_dGPD1dGPD2 strain could grow in the fed-batch fermentation since initial glucose concentration is rather low (100 g/L). Improved maximum DCW and glucose consumption rates resulted in high productivity of 2,3-BD by the BD5_Ctnox_dGPD1dGPD2 strain. While a small amount of acetate accumulated in batch fermentation, no acetate, ethanol, and glycerol were formed in fed-batch fermentation.

In this chapter, through the optimized fed-batch fermentation with the BD5_Ctnox_dGPD1dGPD2 strain which has a deficient glycerol biosynthesis, high yield of 2,3-BD was produced without glycerol formation. The 2,3-BD yield obtained by this system was comparable to bacterial systems with native producers as well as the highest value among the yeast systems for 2,3-BD production. Especially, the utilization of NADH oxidase for relieving cofactor imbalance induced by elimination of the glycerol synthetic pathway could be an attractive strategy for other microbial production systems to control glycerol production.

Table 5-1. Strains and plasmids used in Chapter 5

Strains and plasmids	Description	Reference
Strains		
Saccharomyces cerevisiae D452-2	Source for *ScPDC1*, *ScPDC5, ScPDC6* *MATα leu2 his3 ura3*	(Nikawa et al., 1991)
SOS5	D452-2, *pdc1Δ, pdc5Δ, pdc6Δ*	In this study
BD5	SOS5, p423_alsSalsD, p425_BDH1	In this study
BD5_T2nox	BD5, p426TDH3_Llnox	In this study
BD5_T2nox_dGPD1	BD5, p426TDH3_Llnox *gpd1*	In this study
BD5_T2nox_dGPD2	BD5, p426TDH3_Llnox *gpd2*	In this study
BD5_T2nox_dGPD1dGPD2	BD5, p426TDH3_Llnox *gpd1 gpd2*	In this study
BD5_dGPD1	BD5, *gpd1*	In this study
BD5_dGPD2	BD5, *gpd2*	In this study
BD5_Ctnox_dGPD1dGPD2	BD5, p426TDH3_Llnox *adh1*::*CtPDC1* *gpd1 gpd2*	
Plasmids		
p423_alsSalsD	*HIS3* 2μm origin, $TDH3_{prom}$-*alsS*-$CYC1_{term}$ $TDH3_{prom}$-*alsD*-$CYC1_{term}$	(Kim et al., 2014)
p425_BDH1	*LEU2* 2μm origin, $TDH3_{prom}$-*BDH1*-$CYC1_{term}$	(Kim et al., 2013a)
p426TDH3_Llnox	pRS426, $TDH3_{prom}$-*Llnox*-$CYC1_{term}$	(Kim et al., 2015)
p414_TEF1p_Cas9_CYC1t	*TRP1 CEN6 ARS4* $TEF1_{prom}$-*Cas9*-$CYC1_{term}$	(Zhang et al., 2014)
pAUR_Cas9	*AUR1-C CEN6 ARS4* $TEF1_{prom}$-*Cas9*-$CYC1_{term}$	In this study
pRS42H-gLEU2	Hyg^R gBlock for *LEU2*	(Zhang et al., 2014)

pRS42H-gGPD1	Hyg^R gBlock for *GPD1*	In this study
pRS42H-gGPD2	Hyg^R gBlock for *GPD2*	In this study
pRS42H-gADH1	Hyg^R gBlock for *ADH1*	In this study

Table 5-2. Primers used in Chapter 5. Bold and capital characters are restriction enzyme sites.

Primers	Sequence
Construction of gBlock	
F1_SacI_gBlock	TCTACAGCGGCCGCGAGCTCTCTTTGAAAAGAT AATGTAT
R1_GPD1_gBlock	TGAAAGGCTTTTCGGCAGCCGATCATTTATCTT TCACTGCG
F2_GPD1_gBlock	GGCTGCCGAAAAGCCTTTCAGTTTTAGAGCTAG AAATAGCAAGT
R2_KpnI_gBlock	TATAGAGCGGCCGCGGTACCAGACATAAAAAA CAAAAAAAG
R1_GPD2_gBlock	CATGACAGTGTTTGTGCTGTGATCATTTATCTTT CACTGCG
F2_GPD2_gBlock	ACAGCACAAACACTGTCATGGTTTTAGAGCTAG AAATAGCAAGT
R1_ADH1_gBlock	ACTTACCGTGGGATTCGTAGGATCATTTATCTT TCACTGCG
F2_ADH1_gBlock	CTACGAATCCCACGGTAAGTGTTTTAGAGCTAG AAATAGCAAGT
Constriction of repairDNA	
F3_Repair_GPD1d	AGTTCCTCTTCTGTTTCTTTGAAGGCTGCCGAA AAGCCTTTCAAGTAAACTGTGATTGGA
R3_Repair_GPD1d	CTTGGCAATAGTAGTACCCCAGTTACCAGATCC AATCACAGTTTACTTGAAAGGCTTTTC
F3_Repair_GPD2d	ATGACTGCTCATACTAATATCAAACAGCACAAA CACTGTCATGAGTAACATCCTATCAGA
R3_Repair_GPD2d	ATGTACAATTGACACGGCAGAGTCCGATCTTCT GATAGGATGTTACTCATGACAGTGTTT
F3_Repair_ADH1 _TDH3p	TATACCAAGCATACAATCAACTATCTCATATAC AAGTTTATCATTATCA
R3_Repair_ADH1 _CYC1t	GTAGACAAGCCGACAACCTTGATTGGAGACTT GAGGCCGCAAATTAAAG

Table 5-3. Plasmid curing efficiency of NADH oxidase in the *GPD* deletion strains.

Strains	Nox- cells/Total cells
BD5_T2nox_ dGPD1	5/16 (31.3%)
BD5_T2nox_ dGPD2	6/16 (37.5%)
BD5_T2nox_ dGPD1dGPD2	0/120 (0%)

Table 5-4. Summary of the flask cultivation with *GPD* deletion strains

Parameters	DCW_{max} (g/L)	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
BD5_T2nox	1.3	15.7	1.9	31.5	0.166	0.333	0.33
BD5_T2nox _dGPD1	1.1	6.4	5.6	22.3	0.086	0.302	0.19
BD5_T2nox _dGPD2	1.1	6.4	5.1	24.3	0.083	0.317	0.20
BD5_T2nox _dGPD1dGPD2	1.0	0	1.7	16.5	0	0.363	0.14

Table 5-5. Summary of batch and fed-batch fermentations with the BD5_Ctnox_dGPD1dGPD2 strain

Parameters	Glycerol (g/L)	Acetoin (g/L)	2,3-BD (g/L)	Acetate (g/L)	Ethanol (g/L)	Glycerol yield (g/g)	2,3-BD yield (g/g)	2,3-BD productivity (g/L/h)
Batch	0.5	11.3	99.4	1.3	0.2	0.002	0.418 (83.6%)	0.62
Fed-batch	0.3	4.6	108.6	0.1	0	0.001	0.462 (92.4%)	1.55

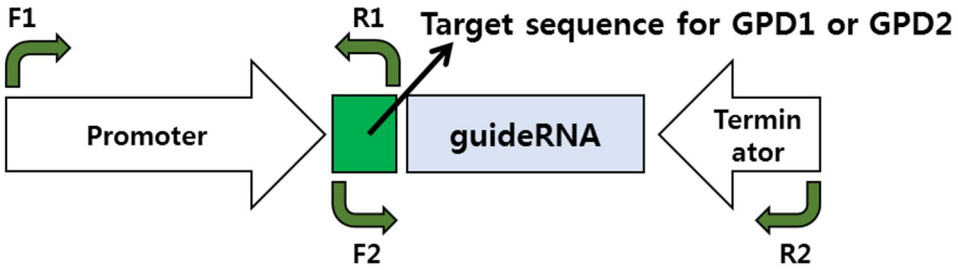


Figure 5-1. Schematic diagram of PCR-based guideRNA expression cassette design for genetic engineering of *GPD1* and *GPD2* gene by Cas9-CRISPR system.

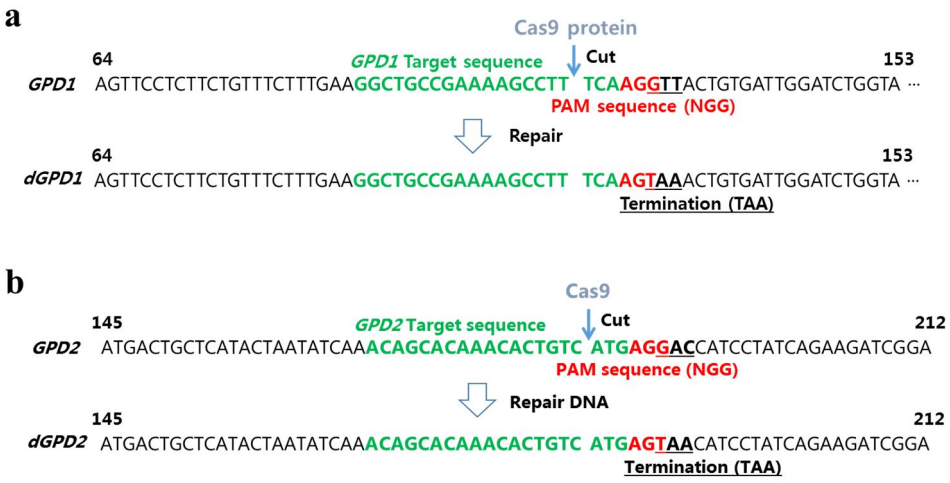
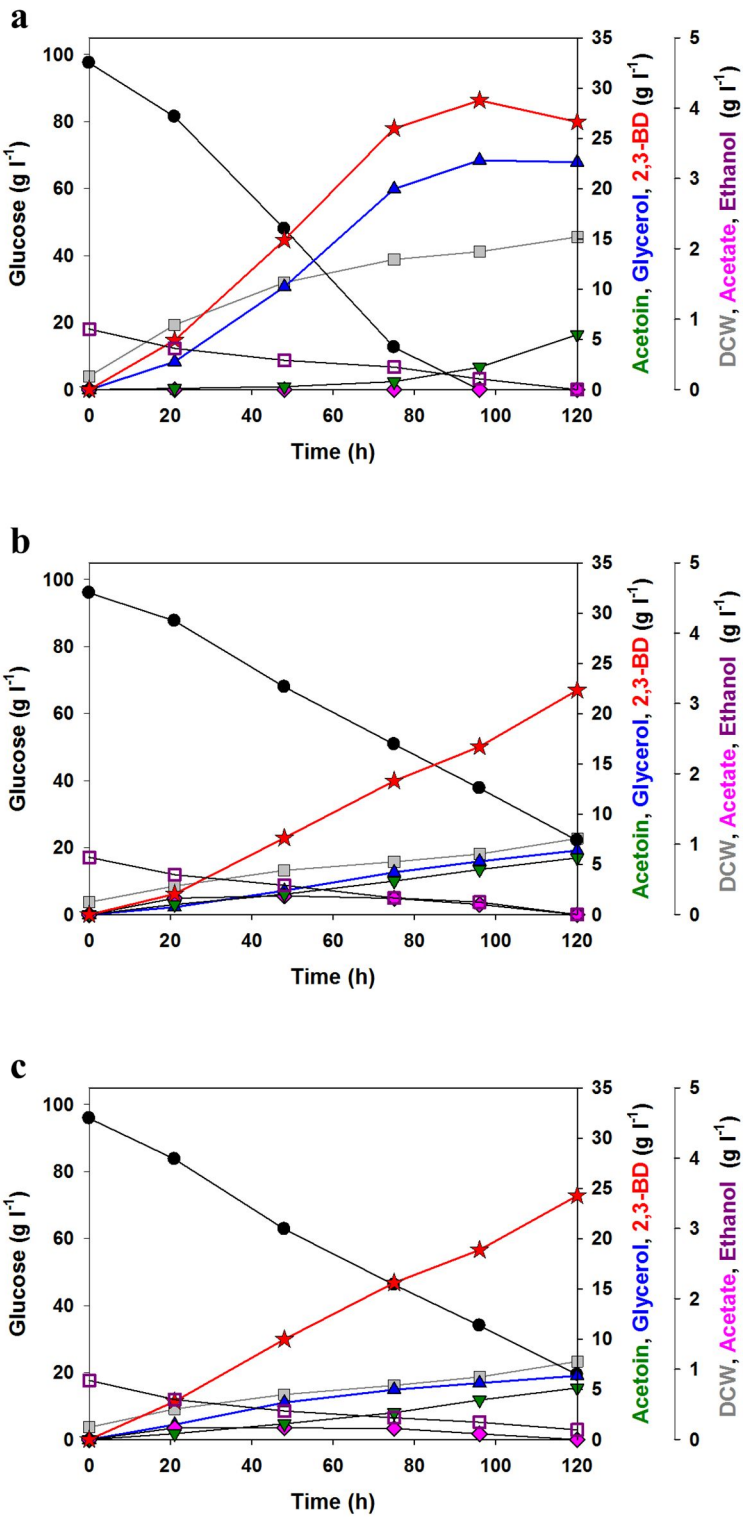


Figure 5-2. Point mutation on (a) *GPD1* and (b) *GPD2* genes by Cas9-CRISPR system.


a
b
c
Glucose (g l-1)
Acetoin, Glycerol, 2,3-BD (g l-1)
DCW, Acetate, Ethanol (g l-1)
Time (h)
0
20
40
60
80
100
120
0
5
10
15
20
25
30
35
0
1
2
3
4
5

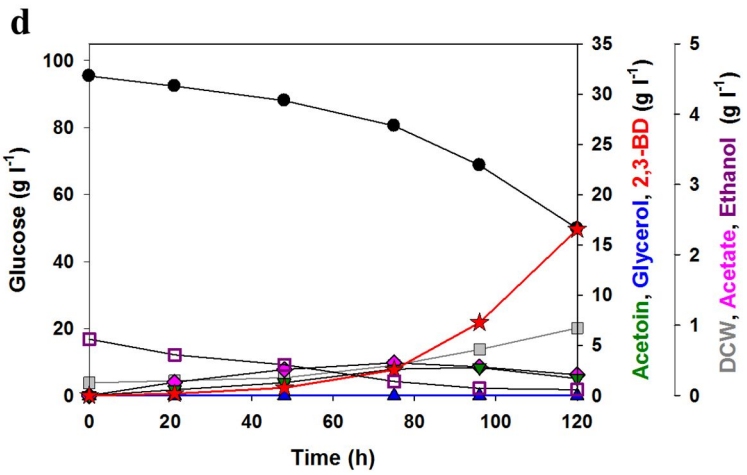


Figure 5-3. Flask cultivation of *GPD* deletion strains. (a), BD5_T2nox; (b), BD5_T2nox_dGPD1; (c), BD5_T2nox_dGPD2; (d), BD5_T2nox_dGPD1dGPD2. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

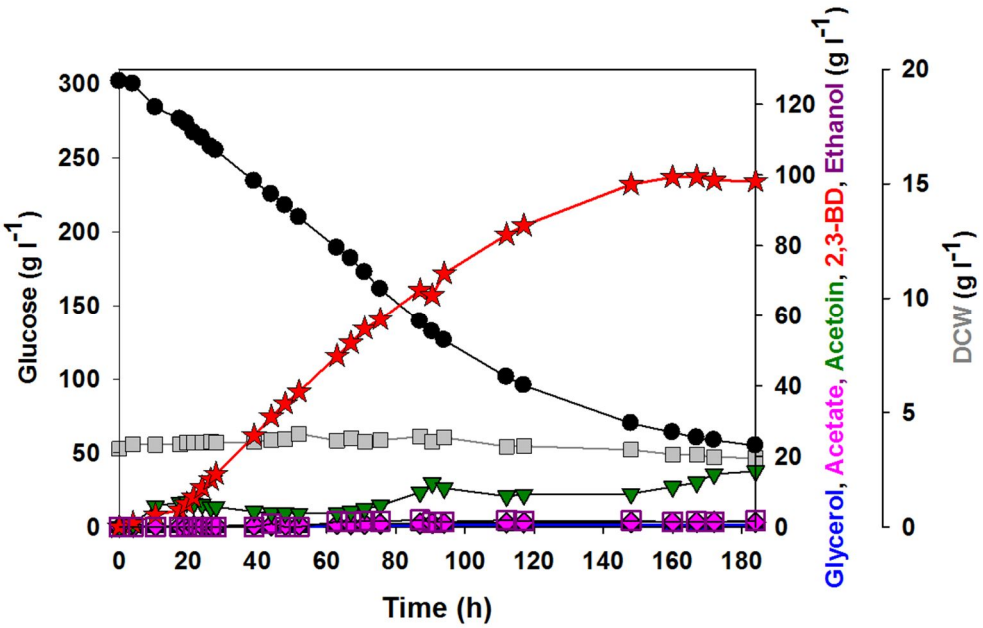


Figure 5-4. Batch fermentation of BD5_Ctnox_dGPD1dGPD2 strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

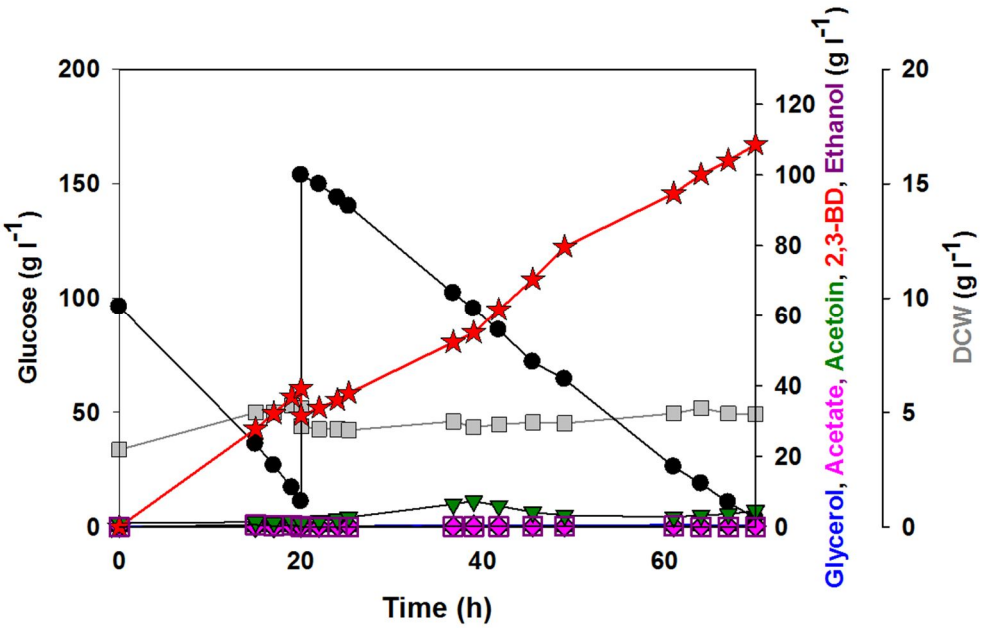


Figure 5-5. Fed-batch fermentation of BD5_Ctnox_dGPD1dGPD2 strain. Symbols: ⊠, DCW ●, glucose; ▲, glycerol; ; ◆, acetate; ▼, acetoin; □, ethanol; ★, 2,3-BD.

Conclusions

Conclusions

2,3-Butanediol (2,3-BD) is a promising chemical that has multiple applications to chemical industries. *S. cerevisiae* is a non-pathogenic GRAS (Generally Recognized as Safe) microorganism which is suitable for large-scale fermentations. The objective of this study is development of an efficient 2,3-BD production system using *S. cerevisiae* as a host strain. To do this, cofactor and pathway engineering and optimization of fermentation processes were applied for maximizing 2,3-BD yield, productivity and titer.

First, in Chapter 2, to switch *S. cerevisiae* from a native ethanol producer to a 2,3-BD-producing strain, the *S. cerevisiae* D452-2 strain was engineered by deletion of pyruvate decarboxylase (Pdc) and introduction of the 2,3-BD biosynthetic pathway. The resulting strain (BD5) could produce 2,3-BD instead of ethanol from glucose. However, the BD5 strain could not produce 2,3-BD efficiently because of i) glycerol accumulation by cofactor imbalance and ii) retarded cell growth by insufficient acetyl-CoA supply.

Second, in Chapter 3, *Lactococcus lactis* NADH oxidase (*noxE*) was expressed for relieving cofactor imbalance in the BD5 strain. Control of intracellular excess NADH by NADH oxidase resulted in improved 2,3-BD production. The resulting BD5_T2nox strain produced 2,3-BD with yield of 0.359 $g_{2,3\text{-BD}}/g_{Glucose}$ 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* strain.

Third, in Chapter 4, Pdc activity was fine-tuned for improving cell growth and carbon consumption rate. Since Pdc-deficient *S. cerevisiae* cannot synthesize enough acetyl-CoA in cytosol, supplementation of a trace amount of C_2-compounds such as an ethanol could significantly increase cell growth and 2,3-BD productivity. However, expression levels of *PDC* should be controlled because pyruvate is a precursor for synthesizing 2,3-BD as well as supplying C_2-compounds. Thus, the 2,3-BD yield and productivity could be maximized by fine-tuned expression of *PDC* through the combination of *PDC* gene source, promoter, and copy numbers. 2,3-BD productivity of the resulting strain (BD5_G1CtPDC1) was improved by 2.4-fold while maintaining the same 2,3-BD yield. The fed-batch fermentation resulted in 121.8 g/L of 2,3-BD in 80 h cultivation. Additionally, the BD5_Ctnox strain co-expressing *PDC* and *noxE* produced 154.3 g/L of 2,3-BD with 1.98 $g_{2,3\text{-}BD}$/L/h of productivity and 0.404 $g_{2,3\text{-}BD}/g_{Glucose}$.

Fourth, in Chapter 5, the glycerol synthetic pathway was removed for increasing 2,3-BD yield. In the *GPD* deletion strains, NADH oxidase acted a role as a redox sink instead of the glycerol production. The *gpd* null mutant strains produced 2,3-BD without glycerol formation. The fed-batch fermentation with the BD5_Ctnox_dGPD1dGPD2 produced 108.6 g/L of 2,3-BD in 76 h of cultivation. The 2,3-BD yield of 0.462 $g_{2,3\text{-}BD}/g_{Glucose}$ is corresponded to 92.4% of theoretical yield of 2,3-BD.

In this study, an ethanologenic *S. cerevisiae* was engineered for the efficient 2,3-BD production strain. High yield, titer, and productivity of 2,3-BD were achieved by fed-batch fermentation with the engineered strains. Especially, 2,3-BD titer of 154.3 g/L is the highest value among other microbial production studies. *S. cerevisiae* is a GRAS microorganism and has no concerns about bacteriophage contamination and biofilm formation which influence on process safety and stability. Since the process safety and stability are the important issues for economic feasibility of microbial production, the 2,3-BD production system with *S. cerevisiae* developed in this study could be superior to the bacterial system for industrial applications. In addition to 2,3-BD production, the cofactor and pathway engineering strategies on Pdc-deficient *S. cerevisiae* could be useful for other research on chemical production by pyruvate as a precursor.

References

Afschar, A., Bellgardt, K., Rossell, C.V., Czok, A., Schaller, K. 1991. The production of 2, 3-butanediol by fermentation of high test molasses. *Appl. Microb. Biotech.*, **34**(5), 582-585.

Albers, E., Larsson, C., Lidén, G., Niklasson, C., Gustafsson, L. 1996. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Appl. Environ. Microbiol.*, **62**(9), 3187-3195.

Albers, E., Lidén, G., Larsson, C., Gustafsson, L. 1998. Anaerobic redox balance and nitrogen metabolism in *Saccharomyces cerevisiae*. *Rec Res Devel in Microbiology*, **2**, 253-279.

Anderlund, M., Nissen, T.L., Nielsen, J., Villadsen, J., Rydström, J., Hahn-Hägerdal, B., Kielland-Brandt, M.C. 1999. Expression of the *Escherichia coli pntA* and *pntB* Genes, Encoding Nicotinamide Nucleotide Transhydrogenase, in *Saccharomyces cerevisiae* and Its Effect on Product Formation during Anaerobic Glucose Fermentation. *Applied and Environmental Microbiology*, **65**(6), 2333-2340.

Andreasen, A., Stier, T. 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty and requirement for growth in a defined medium. *Journal of Cellular and Comparative Physiology*, **43**(3), 271-281.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M., Adler, L. 1997. The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. *EMBO J.*, **16**(9), 2179-2187.

Arjunan, P., Umland, T., Dyda, F., Swaminathan, S., Furey, W., Sax, M., Farrenkopf, B., Gao, Y., Zhang, D., Jordan, F. 1996. Crystal Structure of the Thiamin Diphosphate-dependent Enzyme Pyruvate Decarboxylase from the Yeast *Saccharomyces cerevisiae* at 2.3 Å Resolution. *Journal of Molecular Biology*, **256**(3), 590-600.

Atsumi, S., Li, Z., Liao, J.C. 2009. Acetolactate synthase from *Bacillus subtilis* serves as a 2-ketoisovalerate decarboxylase for isobutanol biosynthesis in *Escherichia coli*. *Appl. Environ. Microbiol.*, **75**(19), 6306-6311.

Baburina, I., Gao, Y., Hu, Z., Jordan, F., Hohmann, S., Furey, W. 1994. Substrate activation of brewers' yeast pyruvate decarboxylase is abolished by mutation of cysteine 221 to serine. *Biochemistry*, **33**(18), 5630-5635.

Bakker, B.M., Overkamp, K.M., Maris, A.J., Kötter, P., Luttik, M.A., Dijken, J.P., Pronk, J.T. 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.*, **25**(1), 15-37.

Bartowsky, E.J., Henschke, P.A. 2004. The 'buttery'attribute of wine—diacetyl—desirability, spoilage and beyond. *International Journal of Food Microbiology*, **96**(3), 235-252.

Biswas, R., Yamaoka, M., Nakayama, H., Kondo, T., Yoshida, K.-i., Bisaria, V.S., Kondo, A. 2012. Enhanced production of 2, 3-butanediol by engineered *Bacillus subtilis*. *Appl. Microb. Biotech.*, **94**(3), 651-658.

Björkqvist, S., Ansell, R., Adler, L., Liden, G. 1997. Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of

Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, **63**(1), 128-132.

Blomberg, A., Larsson, C., Gustafsson, L. 1988. Microcalorimetric monitoring of growth of *Saccharomyces cerevisiae*: osmotolerance in relation to physiological state. *Journal of Bacteriology*, **170**(10), 4562-4568.

Boonstra, B., French, C.E., Wainwright, I., Bruce, N.C. 1999. The *udhA* gene of *Escherichia coli* encodes a soluble pyridine nucleotide transhydrogenase. *Journal of Bacteriology*, **181**(3), 1030-1034.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**(1), 248-254.

Bro, C., Regenberg, B., Förster, J., Nielsen, J. 2006. In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. *Metabolic Engineering*, **8**(2), 102-111.

Bruinenberg, P.M., Jonker, R., van Dijken, J.P., Scheffers, W.A. 1985. Utilization of formate as an additional energy source by glucose-limited chemostat cultures of *Candida utilis* CBS 621 and *Saccharomyces cerevisiae* CBS 8066. *Archives of Microbiology*, **142**(3), 302-306.

Bruinenberg, P.M., Van Dijken, J.P., Scheffers, W.A. 1983a. An enzymic analysis of NADPH production and consumption in *Candida utilis*. *Journal of General Microbiology*, **129**(4), 965-971.

Bruinenberg, P.M., Van Dijken, J.P., Scheffers, W.A. 1983b. A theoretical analysis of NADPH production and consumption in yeasts. *Journal of General Microbiology*, **129**(4), 953-964.

Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D. 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science*, **273**(5278), 1058-1073.

Camougrand, N., Cheyrou, A., Henry, M., Guérin, M. 1988. The alternative respiratory pathway of the yeast *Candida parapsilosis*: oxidation of exogenous NAD(P)H. *Journal of General Microbiology*, **134**(12), 3195-3204.

Cao, N., Xia, Y., Gong, C.S., Tsao, G.T. 1997. Production of 2, 3-butanediol from pretreated corn cob by *Klebsiella oxytoca* in the presence of fungal cellulase. in: *Biotechnology for Fuels and Chemicals*, Springer, pp. 129-139.

Celińska, E., Grajek, W. 2009. Biotechnological production of 2, 3-butanediol—current state and prospects. *Biotechnology Advances*, **27**(6), 715-725.

Chen, C., Wei, D., Shi, J., Wang, M., Hao, J. 2014. Mechanism of 2, 3-butanediol stereoisomer formation in Klebsiella pneumoniae. *Applied microbiology and biotechnology*, **98**(10), 4603-4613.

Cheng, K.-K., Liu, Q., Zhang, J.-A., Li, J.-P., Xu, J.-M., Wang, G.-H. 2010. Improved 2, 3-butanediol production from corncob acid hydrolysate by fed-batch fermentation using *Klebsiella oxytoca*. *Process Biochemistry*, **45**(4), 613-616.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**(6121), 819-823.

Costenoble, R., Valadi, H., Gustafsson, L., Niklasson, C., Johan Franzén, C. 2000. Microaerobic glycerol formation in *Saccharomyces cerevisiae*. *Yeast*, **16**(16), 1483-1495.

De Deken, R. 1966. The Crabtree effect: a regulatory system in yeast. *Journal of General Microbiology*, **44**(2), 149-156.

de Felipe, F.L., Kleerebezem, M., de Vos, W.M., Hugenholtz, J. 1998a. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. *Journal of Bacteriology*, **180**(15), 3804-3808.

De Felipe, F.L., Kleerebezem, M., de Vos, W.M., Hugenholtz, J. 1998b. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. *J. Bacteriol.*, **180**(15), 3804-3808.

Denkert, C., Budczies, J., Weichert, W., Wohlgemuth, G., Scholz, M., Kind, T., Niesporek, S., Noske, A., Buckendahl, A., Dietel, M. 2008. Metabolite profiling of human colon carcinoma–deregulation of TCA cycle and amino acid turnover. *Molecular Cancer*, **7**(1), 72.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., Church, G.M. 2013. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research*, gkt135.

Donker, H.J.L. 1926. Bijdrage tot de kennis der boterzuur-, butylalcohol-en acetongistingen, TU Delft, Delft University of Technology.

Ehsani, M., Fernández, M.R., Biosca, J.A., Julien, A., Dequin, S. 2009. Engineering of 2, 3-butanediol dehydrogenase to reduce acetoin formation

by glycerol-overproducing, low-alcohol *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, **75**(10), 3196-3205.

Eriksson, P., André, L., Ansell, R., Blomberg, A., Adler, L. 1995. Cloning and characterization of *GPD2*, a second gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) in *Saccharomyces cerevisiae*, and its comparison with *GPD1*. *Mol. Microbiol.*, **17**(1), 95-107.

Everse, J. 2012. *The pyridine nucleotide coenzymes*. Elsevier.

Falco, S.C., Dumas, K.S., Livak, K.J. 1985. Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. *Nucleic Acids Research*, **13**(11), 4011-4027.

Fiechter, A., Seghezzi, W. 1992. Regulation of glucose metabolism in growing yeast cells. *Journal of Biotechnology*, **27**(1), 27-45.

Flick, K.M., Spielewoy, N., Kalashnikova, T.I., Guaderrama, M., Zhu, Q., Chang, H.-C., Wittenberg, C. 2003. Grr1-dependent inactivation of Mth1 mediates glucose-induced dissociation of Rgt1 from *HXT* gene promoters. *Molecular Biology of the Cell*, **14**(8), 3230-3241.

Flickinger, M.C. 1980. Current biological research in conversion of cellulosic carbohydrates into liquid fuels: how far have we come. *Biotechnol. Bioeng.*, **22**.

Flikweert, M.T., Swaaf, M., Dijken, J.P., Pronk, J.T. 1999. Growth requirements of pyruvate-decarboxylase-negative *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.*, **174**(1), 73-79.

Flikweert, M.T., van der Zanden, L., Janssen, W.M.T.M., Yde Steensma, H., van Dijken, J.P., Pronk, J.T. 1996. Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. *Yeast*, **12**(3), 247-257.

French, C.E., Boonstra, B., Bufton, K., Bruce, N.C. 1997. Cloning, sequence, and properties of the soluble pyridine nucleotide transhydrogenase of *Pseudomonas fluorescens*. *Journal of Bacteriology*, **179**(8), 2761-2765.

Gancedo, J.M. 1998. Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.*, **62**(2), 334-361.

Gao, J., Xu, H., Li, Q.-j., Feng, X.-h., Li, S. 2010. Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce *R*, *R*-2, 3-butanediol. *Bioresour. Technol.*, **101**(18), 7076-7082.

Garg, S., Jain, A. 1995. Fermentative production of 2, 3-butanediol: a review. *Bioresour. Technol.*, **51**(2), 103-109.

Geertman, J.M.A., Van Dijken, J.P., Pronk, J.T. 2006. Engineering NADH metabolism in *Saccharomyces cerevisiae*: formate as an electron donor for glycerol production by anaerobic, glucose-limited chemostat cultures. *FEMS Yeast Res.*, **6**(8), 1193-1203.

Geueke, B., Riebel, B., Hummel, W. 2003. NADH oxidase from *Lactobacillus brevis*: a new catalyst for the regeneration of NAD. *Enzyme Microb. Technol.*, **32**(2), 205-211.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B. 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *nature*, **418**(6896), 387-391.

Gommers, P., Van Schie, B., Van Dijken, J., Kuenen, J. 1988. Biochemical Limits to Microbial-Growth Yields-an Analysis of Mixed Substrate Utilization. *Biotechnology and Bioengineering*, **1**, 86-94.

González, E., Fernández, M.R., Marco, D., Calam, E., Sumoy, L., Parés, X., Dequin, S., Biosca, J.A. 2010. Role of *Saccharomyces cerevisiae* oxidoreductases Bdh1p and Ara1p in the metabolism of acetoin and 2, 3-butanediol. *Appl. Environ. Microbiol.*, **76**(3), 670-679.

González, S.M., Freire, P.M., Cerdán, M. 1996. Reoxidation of the NADPH produced by the pentose phosphate pathway is necessary for the utilization of glucose by *Kluyveromyces lactis rag2* mutants. *FEBS Letters*, **387**(1), 7-10.

Guo, Z.-p., Zhang, L., Ding, Z.-y., Shi, G.-y. 2011. Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance. *Metabolic engineering*, **13**(1), 49-59.

Guo, Z., Zhang, L., Ding, Z., Wang, Z., Shi, G. 2009. Interruption of glycerol pathway in industrial alcoholic yeasts to improve the ethanol production. *Appl. Microbiol. Biotechnol.*, **82**(2), 287-292.

Guymon, J.F., Crowell, E. 1967. Direct gas chromatographic determination of levo-and meso-2, 3-butanediols in wines and factors affecting their formation. *Am. J. Enol. Vitic.*, **18**(4), 200-209.

Häßler, T., Schieder, D., Pfaller, R., Faulstich, M., Sieber, V. 2012. Enhanced fed-batch fermentation of 2, 3-butanediol by *Paenibacillus polymyxa* DSM 365. *Bioresour. Technol.*, **124**, 237-244.

Han, S.-H., Lee, J.-E., Park, K., Park, Y.-C. 2013. Production of 2, 3-butanediol by a low-acid producing *Klebsiella oxytoca* NBRF4. *New Biotechnology*, **30**(2), 166-172.

Harden, A., Walpole, G.S. 1906. Chemical action of *Bacillus lactis* aerogenes (*Escherich*) on glucose and mannitol: production of 2, 3-butyleneglycol and acetylmethylcarbinol. *Proceedings of the Royal Society of London. Series B*, 399-405.

Hatti-Kaul, R., Törnvall, U., Gustafsson, L., Börjesson, P. 2007. Industrial biotechnology for the production of bio-based chemicals–a cradle-to-grave perspective. *Trends in Biotechnology*, **25**(3), 119-124.

Haveren, J.v., Scott, E.L., Sanders, J. 2008. Bulk chemicals from biomass. *Biofuels, Bioproducts and Biorefining*, **2**(1), 41-57.

Heux, S., Cachon, R., Dequin, S. 2006. Cofactor engineering in *Saccharomyces cerevisiae*: Expression of a H_2O-forming NADH oxidase and impact on redox metabolism. *Metab. Eng.*, **8**(4), 303-314.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B.-C., Herrmann, R. 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Research*, **24**(22), 4420-4449.

Hoek, J.B., Rydstrom, J. 1988. Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem. J*, **254**, 1-10.

Hohmann, S. 1991. Characterization of *PDC6*, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. *J. Biotechnol.*, **173**(24), 7963-7969.

Hohmann, S., Cederberg, H. 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. *Eur. J. Biochem.*, **188**(3), 615-621.

Hoskins, D.D., Whiteley, H., Mackler, B. 1962. The reduced diphosphopyridine nucleotide oxidase of *Streptococcus faecalis*: purification and properties. *J. Biol. Chem.*, **237**(8), 2647-2651.

Hou, J., Lages, N.F., Oldiges, M., Vemuri, G.N. 2009a. Metabolic impact of redox cofactor perturbations in *Saccharomyces cerevisiae*. *Metab. Eng.*, **11**(4), 253-261.

Hou, J., Suo, F., Wang, C., Li, X., Shen, Y., Bao, X. 2014. Fine-tuning of NADH oxidase decreases byproduct accumulation in respiration deficient xylose metabolic *Saccharomyces cerevisiae*. *BMC Biotechnology*, **14**(1), 13.

Hou, J., Vemuri, G.N., Bao, X., Olsson, L. 2009b. Impact of overexpressing NADH kinase on glucose and xylose metabolism in recombinant xylose-utilizing *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*, **82**(5), 909-919.

Hubmann, G., Guillouet, S., Nevoigt, E. 2011. Gpd1 And Gpd2 fine-tuning for sustainable reduction of glycerol formation in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, **77**(17), 5857-5867.

Hummel, W., Riebel, B. 2003. Isolation and biochemical characterization of a new NADH oxidase from *Lactobacillus brevis*. *Biotechnology Letters*, **25**(1), 51-54.

Ishida, N., Saitoh, S., Onishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K., Takahashi, H. 2006. The effect of pyruvate decarboxylase gene knockout in *Saccharomyces cerevisiae* on L-lactic acid production. *Biosci. Biotechnol. Biochem.*, **70**(5), 1148-1153.

Jagow, G., Klingenberg, M. 1970. Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. *European Journal of Biochemistry*, **12**(3), 583-592.

Jantama, K., Polyiam, P., Khunnonkwao, P., Chan, S., Sangproo, M., Khor, K., Jantama, S.S., Kanchanatawee, S. 2015. Efficient reduction of the formation of by-products and improvement of production yield of 2, 3-butanediol by a combined deletion of alcohol dehydrogenase, acetate kinase-phosphotransacetylase, and lactate dehydrogenase genes in metabolically engineered *Klebsiella oxytoca* in mineral salts medium. *Metabolic Engineering*, **30**, 16-26.

Jeun, Y.-S., Kim, M.-D., Park, Y.-C., Lee, T.-H., Yoo, M.-S., Ryu, Y.-W., Seo, J.-H. 2003. Expression of *Azotobacter vinelandii* soluble transhydrogenase perturbs xylose reductase-mediated conversion of xylose to xylitol by recombinant *Saccharomyces cerevisiae*. *Journal of Molecular Catalysis B: Enzymatic*, **26**(3), 251-256.

Ji, X.-J., Huang, H., Ouyang, P.-K. 2011. Microbial 2, 3-butanediol production: a state-of-the-art review. *Biotechnology Advances*, **29**(3), 351-364.

Ji, X.-J., Huang, H., Zhu, J.-G., Ren, L.-J., Nie, Z.-K., Du, J., Li, S. 2010. Engineering *Klebsiella oxytoca* for efficient 2, 3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. *Appl. Microbiol. Biotechnol.*, **85**(6), 1751-1758.

Jia, B., Park, S.C., Lee, S., Pham, B.P., Yu, R., Le, T.L., Han, S.W., Yang, J.K., Choi, M.S., Baumeister, W. 2008. Hexameric ring structure of a thermophilic archaeon NADH oxidase that produces predominantly H_2O. *FEBS Journal*, **275**(21), 5355-5366.

John, R.P., Nampoothiri, K.M., Pandey, A. 2007. Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives. *Applied Microbiology and Biotechnology*, **74**(3), 524-534.

Kamm, B., Kamm, M. 2004. Principles of biorefineries. *Appl. Microbiol. Biotechnol.*, **64**(2), 137-145.

Kappeli, O. 1986. Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Adv Microb Physiol*, **28**, 181-209.

Kawai, S., Suzuki, S., Mori, S., Murata, K. 2001. Molecular cloning and identification of *UTR1* of a yeast *Saccharomyces cerevisiae* as a gene encoding an NAD kinase. *FEMS Microbiology Letters*, **200**(2), 181-184.

Kawasaki, S., Ishikura, J., Chiba, D., Nishino, T., Niimura, Y. 2004. Purification and characterization of an H_2O-forming NADH oxidase from *Clostridium aminovalericum*: existence of an oxygen-detoxifying enzyme in an obligate anaerobic bacteria. *Archives of Microbiology*, **181**(4), 324-330.

Kim, J.-H. 2009. DNA-binding properties of the yeast Rgt1 repressor. *Biochimie*, **91**(2), 300-303.

Kim, J.-H., Brachet, V., Moriya, H., Johnston, M. 2006. Integration of transcriptional and posttranslational regulation in a glucose signal transduction pathway in *Saccharomyces cerevisiae*. *Eukaryotic Cell*, **5**(1), 167-173.

Kim, J.-W., Chin, Y.-W., Park, Y.-C., Seo, J.-H. 2012. Effects of deletion of glycerol-3-phosphate dehydrogenase and glutamate dehydrogenase genes on glycerol and ethanol metabolism in recombinant *Saccharomyces cerevisiae*. *Bioprocess and Biosystems Engineering*, **35**(1-2), 49-54.

Kim, J.-W., Seo, S.-O., Zhang, G.-C., Jin, Y.-S., Seo, J.-H. 2015. Expression of *Lactococcus lactis* NADH oxidase increases 2, 3-butanediol production in Pdc-deficient *Saccharomyces cerevisiae*. *Bioresour. Technol.*, **191**, 512-519.

Kim, S.-B., Timmusk, S. 2013. A simplified method for gene knockout and direct screening of recombinant clones for application in *Paenibacillus polymyxa*. *PloS one*, **8**(6), e68092.

Kim, S.-J., Seo, S.-O., Jin, Y.-S., Seo, J.-H. 2013a. Production of 2, 3-butanediol by engineered *Saccharomyces cerevisiae*. *Bioresour. Technol.*, **146**, 274-281.

Kim, S.-J., Seo, S.-O., Park, Y.-C., Jin, Y.-S., Seo, J.-H. 2014. Production of 2, 3-butanediol from xylose by engineered *Saccharomyces cerevisiae*. *J. Biotechnol.*, **192**, 374-382.

Kim, S., Hahn, J.-S. 2015. Efficient production of 2, 3-butanediol in Saccharomyces cerevisiae by eliminating ethanol and glycerol production and redox rebalancing. *Metabolic engineering*, **31**, 94-101.

Kim, S., Lee, D.Y., Wohlgemuth, G., Park, H.S., Fiehn, O., Kim, K.H. 2013b. Evaluation and optimization of metabolome sample preparation methods for *Saccharomyces cerevisiae*. *Analytical chemistry*, **85**(4), 2169-2176.

Koike, K., Kobayashi, T., Susumu, I., Saitoh, M. 1985. Purification and characterization of NADH oxidase from a strain of *Leuconostoc mesenteroides*. *J. Biochem.*, **97**(5), 1279-1288.

Kong, Q.-X., Gu, J.-G., Cao, L.-M., Zhang, A.-L., Chen, X., Zhao, X.-M. 2006. Improved production of ethanol by deleting *FPS1* and over-expressing *GLT1* in *Saccharomyces cerevisiae*. *Biotechnology Letters*, **28**(24), 2033-2038.

Kong, Q.-X., Zhang, A.-L., Cao, L.-M., Chen, X. 2007. Over-expressing *GLT1* in a *gpd2Δ* mutant of *Saccharomyces cerevisiae* to improve ethanol production. *Applied Microbiology and Biotechnology*, **75**(6), 1361-1366.

López, J., Thoms, B., Rehbein, H. 1975. Acetoin degradation in *Bacillus subtilis* by direct oxidative cleavage. *Eur. J. Biochem.*, **57**(2), 425-430.

Lafuente, M.J., Gancedo, C., Jauniaux, J.C., Gancedo, J.M. 2000. Mth1 receives the signal given by the glucose sensors Snf3 and Rgt2 in *Saccharomyces cerevisiae*. *Mol. Microbiol.*, **35**(1), 161-172.

Larsson, C., Påhlman, I.L., Ansell, R., Rigoulet, M., Adler, L., Gustafsson, L. 1998. The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. *Yeast*, **14**(4), 347-357.

Lee, D.Y., Fiehn, O. 2008. High quality metabolomic data for *Chlamydomonas reinhardtii*. *Plant Methods*, **4**(1), 7.

Lerner, F., Niere, M., Ludwig, A., Ziegler, M. 2001. Structural and functional characterization of human NAD kinase. *Biochemical and biophysical research communications*, **288**(1), 69-74.

Li, C., Lesnik, K.L., Liu, H. 2013. Microbial Conversion of Waste Glycerol from Biodiesel Production into Value-Added Products. *Energies*, **6**(9), 4739-4768.

Li, L., Li, K., Wang, Y., Chen, C., Xu, Y., Zhang, L., Han, B., Gao, C., Tao, F., Ma, C. 2015. Metabolic engineering of *Enterobacter cloacae* for high-yield production of enantiopure (2R, 3R)-2, 3-butanediol from lignocellulose-derived sugars. *Metabolic Engineering*, **28**, 19-27.

Li, Z.-J., Jian, J., Wei, X.-X., Shen, X.-W., Chen, G.-Q. 2010. Microbial production of meso-2, 3-butanediol by metabolically engineered *Escherichia coli* under low oxygen condition. *Applied Microbiology and Biotechnology*, **87**(6), 2001-2009.

Lian, J., Chao, R., Zhao, H. 2014. Metabolic engineering of a *Saccharomyces cerevisiae* strain capable of simultaneously utilizing glucose and galactose to produce enantiopure (*2R*, *3R*)-butanediol. *Metab. Eng.*, **23**, 92-99.

Liu, Z., Qin, J., Gao, C., Hua, D., Ma, C., Li, L., Wang, Y., Xu, P. 2011. Production of (2S, 3S)-2, 3-butanediol and (3S)-acetoin from glucose using resting cells of *Klebsiella pneumonia* and *Bacillus subtilis*. *Bioresour. Technol.*, **102**(22), 10741-10744.

Lopez de Felipe, F., Hugenholtz, J. 2001. Purification and characterisation of the water forming NADH-oxidase from *Lactococcus lactis*. *International Dairy Journal*, **11**(1), 37-44.

Lunt, S.Y., Vander Heiden, M.G. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annual Review of Cell and Developmental Biology*, **27**, 441-464.

Luttik, M.A., Overkamp, K.M., Kötter, P., de Vries, S., van Dijken, J.P., Pronk, J.T. 1998. The *Saccharomyces cerevisiae NDE1* and *NDE2* genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. *Journal of Biological Chemistry*, **273**(38), 24529-24534.

Ma, C., Wang, A., Qin, J., Li, L., Ai, X., Jiang, T., Tang, H., Xu, P. 2009. Enhanced 2, 3-butanediol production by *Klebsiella pneumoniae* SDM. *Appl. Microbiol. Biotechnol.*, **82**(1), 49-57.

Maddox, I.S. 1996. Microbial Production of 2, 3-Butanediol. *Biotechnology Set, Second Edition*, 269-291.

Magee, R.J., Kosaric, N. 1987. The microbial production of 2, 3-butanediol. *Adv. Appl. Microbiol*, **32**(1), 89-161.

Mali, P., Esvelt, K.M., Church, G.M. 2013a. Cas9 as a versatile tool for engineering biology. *Nature Methods*, **10**(10), 957-963.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M. 2013b. RNA-guided human genome engineering via Cas9. *Science*, **339**(6121), 823-826.

Marks, T., Sharp, R. 2000. Bacteriophages and biotechnology: a review. *Journal of Chemical Technology and Biotechnology*, **75**(1), 6-17.

Masako, I., Ishibashi, S. 1979. Functioning of mitochondria-bound hexokinase in rat brain in accordance with generation of ATP inside the organelle. *Journal of Biochemistry*, **85**(5), 1151-1156.

Matsumoto, J., Higuchi, M., Shimada, M., Yamamoto, Y., Kamio, Y. 1996. Molecular cloning and sequence analysis of the gene encoding the H_2O-forming NADH oxidase from *Streptococcus mutans*. *Biosci. Biotechnol. Biochem.*, **60**(1), 39-43.

Mattila-Sandholm, T., Wirtanen, G. 1992. Biofilm formation in the industry: a review. *Food Reviews International*, **8**(4), 573-603.

McGuinness, E., Butler, J. 1985. NAD^+ kinase—a review. *International Journal of Biochemistry*, **17**(1), 1-11.

Medina, V.G., Almering, M.J., van Maris, A.J., Pronk, J.T. 2010. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl. Environ. Microbiol.*, **76**(1), 190-195.

Michnick, S., Roustan, J., Remize, F., Barre, P., Dequin, S. 1997a. Modulation of glycerol and ethanol yields during alcoholic fermentation in Saccharomyces cerevisiae strains overexpressed or disrupted for GPD1 encoding glycerol 3-phosphate dehydrogenase. *Yeast*, **13**(9), 783-793.

Michnick, S., Roustan, J.L., Remize, F., Barre, P., Dequin, S. 1997b. Modulation of Glycerol and Ethanol Yields During Alcoholic Fermentation in Saccharomyces cerevisiae Strains Overexpressed or Disrupted for GPD1 Encoding Glycerol 3-Phosphate Dehydrogenase. *Yeast*, **13**(9), 783-793.

Moore, C.L., Jöbsis, F.F. 1970. Some studies on the control of respiration in rat brain mitochondrial preparations. *Archives of Biochemistry and Biophysics*, **138**(1), 295-305.

Moreira dos Santos, M., Raghevendran, V., Kötter, P., Olsson, L., Nielsen, J. 2004. Manipulation of malic enzyme in *Saccharomyces cerevisiae* for increasing NADPH production capacity aerobically in different cellular compartments. *Metab. Eng.*, **6**(4), 352-363.

Moriya, H., Johnston, M. 2004a. Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. *Proceedings of the National Academy of Sciences of the United States of America*, **101**(6), 1572-1577.

Moriya, H., Johnston, M. 2004b. Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. *Proc. Natl. Acad. Sci. U.S.A.*, **101**(6), 1572-1577.

Mumberg, D., Müller, R., Funk, M. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, **156**(1), 119-122.

Nan, H., Seo, S.-O., Oh, E.J., Seo, J.-H., Cate, J.H., Jin, Y.-S. 2014. 2, 3-Butanediol production from cellobiose by engineered *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, **98**, 5757-5764.

Navarrete, C., Nielsen, J., Siewers, V. 2014. Enhanced ethanol production and reduced glycerol formation in mutants of engineered for improved redox balancing. *AMB Express*, **1**(4), 1-8.

Nevoigt, E., Stahl, U. 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, **21**(3), 231-241.

Ng, C.Y., Jung, M.-y., Lee, J., Oh, M.-K. 2012. Production of 2, 3-butanediol in *Saccharomyces cerevisiae* by *in silico* aided metabolic engineering. *Microb. Cell Fact.*, **11**, 68.

Nikawa, J.-I., Tsukagoshi, Y., Yamashita, S. 1991. Isolation and characterization of two distinct myo-inositol transporter genes of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **266**(17), 11184-11191.

Nilegaonkar, S., Bhosale, S., Kshirsagar, D., Kapadi, A. 1992. Production of 2, 3-butanediol from glucose by *Bacillus licheniformis*. *World Journal of Microbiology and Biotechnology*, **8**(4), 378-381.

Nissen, T., Hamann, C., Kielland-Brandt, M., Nielsen, J., Villadsen, J. 2000a. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. *Yeast*, **16**(5).

Nissen, T.L., Anderlund, M., Nielsen, J., Villadsen, J., Kielland-Brandt, M.C. 2001. Expression of a cytoplasmic transhydrogenase in *Saccharomyces cerevisiae* results in formation of 2-oxoglutarate due to depletion of the NADPH pool. *Yeast*, **18**(1), 19-32.

Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J. 2000b. Anaerobic and aerobic batch cultivations of Saccharomyces cerevisiae mutants impaired in glycerol synthesis. *Yeast*, **16**(5), 463-474.

Nissen, T.L., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J. 2000c. Optimization of ethanol production in *Saccharomyces cerevisiae* by

metabolic engineering of the ammonium assimilation. *Metab. Eng.*, **2**(1), 69-77.

Nissen, T.L., Schulze, U., Nielsen, J., Villadsen, J. 1997. Flux distributions in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *Microbiology*, **143**(1), 203-218.

Nordström, K. 1968. Yeast growth and glycerol formation II. Carbon and redox balances. *Journal of the Institute of Brewing*, **74**(5), 429-432.

Ostergaard, S., Olsson, L., Nielsen, J. 2000. Metabolic engineering of *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, **64**(1), 34-50.

Oud, B., Flores, C.-L., Gancedo, C., Zhang, X., Trueheart, J., Daran, J.-M., Pronk, J.T., van Maris, A. 2012. An internal deletion in *MTH1* enables growth on glucose of pyruvate-decarboxylase negative, non-fermentative *Saccharomyces cerevisiae*. *Microb. Cell Fact.*, **11**, 131.

Oura, E. 1977. Reaction products of yeast fermentations. *Process Biochemistry*.

Outten, C.E., Culotta, V.C. 2003. A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*. *The EMBO journal*, **22**(9), 2015-2024.

Paciorek-Sadowska, J., Czupryński, B. 2006. New compounds for production of polyurethane foams. *J. Appl. Polym. Sci.*, **102**(6), 5918-5926.

Perego, P., Converti, A., Del Borghi, A., Canepa, P. 2000. 2, 3-Butanediol production by *Enterobacter aerogenes*: selection of the optimal conditions

and application to food industry residues. *Bioprocess Engineering*, **23**(6), 613-620.

Petrik, M., Käppeli, O., Fiechter, A. 1983. An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short-and long-term regulation. *Journal of General Microbiology*, **129**(1), 43-49.

Petrini, P., De Ponti, S., Fare, S., Tanzi, M. 1999. Polyurethane-maleamides for cardiovascular applications: synthesis and properties. *Journal of Materials Science: Materials in Medicine*, **10**(12), 711-714.

Petrov, K., Petrova, P. 2009. High production of 2, 3-butanediol from glycerol by *Klebsiella pneumoniae* G31. *Appl. Microb. Biotech.*, **84**(4), 659-665.

Pohl, M. 1997. Protein design on pyruvate decarboxylase (*PDC*) by site-directed mutagenesis. in: *New Enzymes for Organic Synthesis*, Springer, pp. 15-43.

Polish, J.A., Kim, J.-H., Johnston, M. 2005. How the Rgt1 transcription factor of *Saccharomyces cerevisiae* is regulated by glucose. *Genetics*, **169**(2), 583-594.

Postma, E., Verduyn, C., Scheffers, W.A., Van Dijken, J.P. 1989. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, **55**(2), 468-477.

Pronk, J.T., Steensma, H.Y., Van Dijken, J.P. 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast*, **12**(16), 1607-1633.

Pronk, J.T., Wenzel, T.J., Luttik, M.A., Klaassen, C.C., Scheffers, W.A., Steensma, H.Y., van Dijken, J.P. 1994. Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*. *Microbiology*, **140**(3), 601-610.

Qi, G., Kang, Y., Li, L., Xiao, A., Zhang, S., Wen, Z., Xu, D., Chen, S. 2014. Deletion of meso-2, 3-butanediol dehydrogenase gene budC for enhanced D-2, 3-butanediol production in *Bacillus licheniformis*. *Biotechnology for Biofuels*, **7**(1), 16.

Qureshi, N., Cheryan, M. 1989. Production of 2, 3-butanediol by *Klebsiella oxytoca*. *Appl. Microb. Biotech.*, **30**(5), 440-443.

Rønnow, B., Kielland-Brandt, M.C. 1993. *GUT2*, a gene for mitochondrial glycerol 3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. *Yeast*, **9**(10), 1121-1130.

Riebel, B.R., Gibbs, P.R., Wellborn, W.B., Bommarius, A.S. 2003. Cofactor regeneration of both NAD^+ from NADH and $NADP^+$ from NADPH: NADH oxidase from *Lactobacillus sanfranciscensis*. *Advanced Synthesis & Catalysis*, **345**(6-7), 707-712.

Rigoulet, M., Aguilaniu, H., Avéret, N., Bunoust, O., Camougrand, N., Grandier-Vazeille, X., Larsson, C., Pahlman, I.-L., Manon, S., Gustafsson, L. 2004. Organization and regulation of the cytosolic NADH metabolism in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biochem.*, **256**(1-2), 73-81.

Roca, C., Nielsen, J., Olsson, L. 2003. Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. *Applied and Environmental Microbiology*, **69**(8), 4732-4736.

Romano, P., Suzzi, G. 1996. Origin and production of acetoin during wine yeast fermentation. *Appl. Environ. Microbiol.*, **62**(2), 309.

Ross, R.P., Claiborne, A. 1992. Molecular cloning and analysis of the gene encoding the NADH oxidase from *Streptococcus faecalis* 10C1: Comparison with NADH peroxidase and the flavoprotein disulfide reductases. *J. Mol. Biol.*, **227**(3), 658-671.

Rydstrom, J., Hoek, J., Ernster, L. 1976. The Enzymes (Boyer, PD, ed) Vol. XIII, Academic Press, Inc., New York.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wei, H.-X., Paré, P.W., Kloepper, J.W. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, **100**(8), 4927-4932.

Salusjärvi, L., Kaunisto, S., Holmström, S., Vehkomäki, M.-L., Koivuranta, K., Pitkänen, J.-P., Ruohonen, L. 2013. Overexpression of NADH-dependent fumarate reductase improves D-xylose fermentation in recombinant *Saccharomyces cerevisiae*. *Journal of Industrial Microbiology and Biotechnology*, 1-10.

Schmidt, H.L., Stöcklein, W., Danzer, J., Kirch, P., Limbach, B. 1986. Isolation and properties of an H_2O-forming NADH oxidase from *Streptococcus faecalis*. *Eur. J. Biochem.*, **156**(1), 149-155.

Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*, **343**(6166), 84-87.

Shi, F., Kawai, S., Mori, S., Kono, E., Murata, K. 2005. Identification of ATP-NADH kinase isozymes and their contribution to supply of NADP(H) in *Saccharomyces cerevisiae*. *FEBS Journal*, **272**(13), 3337-3349.

Shianna, K.V., Marchuk, D.A., Strand, M.K. 2006. Genomic characterization of *POS5*, the *Saccharomyces cerevisiae* mitochondrial NADH kinase. *Mitochondrion*, **6**(2), 99-106.

Simion, F.A., Abrutyn, E.S., Draelos, Z.D. 2005. Ability of moisturizers to reduce dry skin and irritation and to prevent their return. *Journal of Cosmetic Science*, **56**(6), 427.

Small, W.C., McAlister-Henn, L. 1998. Identification of a cytosolically directed NADH dehydrogenase in mitochondria of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **180**(16), 4051-4055.

Sponholz, W., Dittrich, H., Muno, H. 1993. Diols in wine. *Vitic. Enol. Sci*, **49**, 23-26.

Sprague, G., Cronan, J. 1977. Isolation and characterization of *Saccharomyces cerevisiae* mutants defective in glycerol catabolism. *Journal of Bacteriology*, **129**(3), 1335-1342.

Strand, M.K., Stuart, G.R., Longley, M.J., Graziewicz, M.A., Dominick, O.C., Copeland, W.C. 2003. *POS5* gene of *Saccharomyces cerevisiae* encodes a mitochondrial NADH kinase required for stability of mitochondrial DNA. *Eukaryotic Cell*, **2**(4), 809-820.

Su, W., Xiao, W.-H., Wang, Y., Liu, D., Zhou, X., Yuan, Y.-J. 2015. Alleviating Redox Imbalance Enhances 7-Dehydrocholesterol Production in Engineered *Saccharomyces cerevisiae*. *PloS one*, **10**(6).

Suga, H., Matsuda, F., Hasunuma, T., Ishii, J., Kondo, A. 2013. Implementation of a transhydrogenase-like shunt to counter redox imbalance during xylose fermentation in Saccharomyces cerevisiae. *Applied microbiology and biotechnology*, **97**(4), 1669-1678.

Suihko, M.-L., Blomqvist, K., Penttilä, M., Gisler, R., Knowles, J. 1990. Recombinant brewer's yeast strains suitable for accelerated brewing. *J. Biotechnol.*, **14**(3), 285-300.

Sun, L.-H., Wang, X.-D., Dai, J.-Y., Xiu, Z.-L. 2009. Microbial production of 2, 3-butanediol from Jerusalem artichoke tubers by *Klebsiella pneumoniae*. *Appl. Microb. Biotech.*, **82**(5), 847-852.

Syu, M.-J. 2001. Biological production of 2, 3-butanediol. *Appl. Microbiol. Biotechnol.*, **55**(1), 10-18.

Tran, A.V., Chambers, R.P. 1987. The dehydration of fermentative 2, 3-butanediol into methyl ethyl ketone. *Biotechnol. Bioeng.*, **29**(3), 343-351.

Ui, S., Takusagawa, Y., Sato, T., Ohtsuki, T., Mimura, A., Ohkuma, M., Kudo, T. 2004. Production of l-2, 3-butanediol by a new pathway constructed in *Escherichia coli*. *Letters in Applied Microbiology*, **39**(6), 533-537.

Valadi, H., Larsson, C., Gustafsson, L. 1998a. Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. *Appl. Microb. Biotech.*, **50**(4), 434-439.

Valadi, H., Larsson, C., Gustafsson, L. 1998b. Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, **50**(4), 434-439.

van Dijken, J.P., Scheffers, W.A. 1986. Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol. Lett.*, **32**(3), 199-224.

van Maris, A.J., Geertman, J.-M.A., Vermeulen, A., Groothuizen, M.K., Winkler, A.A., Piper, M.D., van Dijken, J.P., Pronk, J.T. 2004. Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C_2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. *Appl. Environ. Microbiol.*, **70**(1), 159-166.

van Maris, A.J., Luttik, M.A., Winkler, A.A., van Dijken, J.P., Pronk, J.T. 2003. Overproduction of threonine aldolase circumvents the biosynthetic role of pyruvate decarboxylase in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, **69**(4), 2094-2099.

Van Roermund, C., Elgersma, Y., Singh, N., Wanders, R., Tabak, H. 1995. The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD (H) and acetyl-CoA under in vivo conditions. *The EMBO journal*, **14**(14), 3480.

Van Urk, H., Bruinenberg, P.M., Veenhuis, M., Scheffers, W.A., Van Dijken, J.P. 1989. Respiratory capacities of mitochondria of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 grown under glucose limitation. *Antonie van Leeuwenhoek*, **56**(3), 211-220.

van Urk, H., Voll, W.L., Scheffers, W.A., Van Dijken, J.P. 1990. Transient-state analysis of metabolic fluxes in Crabtree-positive and Crabtree-negative yeasts. *Appl. Environ. Microbiol.*, **56**(1), 281-287.

Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K., Derdelinckx, G., Verachtert, H. 2003. Bioflavoring and beer refermentation. *Appl. Microb. Biotech.*, **62**(2-3), 140-150.

Vemuri, G., Eiteman, M., McEwen, J., Olsson, L., Nielsen, J. 2007. Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.*, **104**(7), 2402-2407.

Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P. 1990. Physiology of *Saccharomyces cerevisiae* in Anaerobic Glucose-Limited Chemostat Cultures. *Journal of General Microbiology*, **136**(3), 395-403.

Verho, R., Londesborough, J., Penttilä, M., Richard, P. 2003. Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, **69**(10), 5892-5897.

Verho, R., Richard, P., Jonson, P.H., Sundqvist, L., Londesborough, J., Penttilä, M. 2002. Identification of the first fungal NADP-GAPDH from *Kluyveromyces lactis*. *Biochemistry*, **41**(46), 13833-13838.

Voloch M, J.N., Ladish MR, Tsao GT, Narayan R, Rodwell VW. 1985. *Comprehensive Biotechnology: the Principles, Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine*. Pergamon/Elsevier.

Wêgrzyn, G. 2004. Bacteriophage contamination: is there a simple method to reduce its deleterious effects in laboratory cultures and biotechnological factories? *J. Appl. Genet*, **45**(1), 111-120.

Walsh, M.C., Scholte, M., Valkier, J., Smits, H.P., van Dam, K. 1996. Glucose sensing and signalling properties in *Saccharomyces cerevisiae* require the presence of at least two members of the glucose transporter family. *J. Bacteriol.*, **178**(9), 2593-2597.

Wang, A., Wang, Y., Jiang, T., Li, L., Ma, C., Xu, P. 2010. Production of 2, 3-butanediol from corncob molasses, a waste by-product in xylitol production. *Appl. Microb. Biotech.*, **87**(3), 965-970.

Wang, L., Chong, H., Jiang, R. 2012a. Comparison of alkyl hydroperoxide reductase and two water-forming NADH oxidases from *Bacillus cereus* ATCC 14579. *Applied Microbiology and Biotechnology*, **96**(5), 1265-1273.

Wang, Z., Gao, C., Wang, Q., Liang, Q., Qi, Q. 2012b. Production of pyruvate in *Saccharomyces cerevisiae* through adaptive evolution and rational cofactor metabolic engineering. *Biochem. Eng. J.*, **67**, 126-131.

Wang, Z., Zhuge, J., Fang, H., Prior, B.A. 2001. Glycerol production by microbial fermentation: a review. *Biotechnol. Adv.*, **19**(3), 201-223.

Wilson, J.E., Felgner, P.L. 1977. An inverse relation between mitochondrial hexokinase content and phosphoglucomutase activity of rat tissues. *Molecular and Cellular Biochemistry*, **18**(1), 39-47.

Winfield, M. 1945. The catalytic dehydration of 2, 3-butanediol to butadiene. *Australian Journal of Scientific Research. Series A.*, **3**(2), 290-305.

Xiu, Z.-L., Zeng, A.-P. 2008. Present state and perspective of downstream processing of biologically produced 1, 3-propanediol and 2, 3-butanediol. *Applied Microbiology and Biotechnology*, **78**(6), 917-926.

Xu, Y., Chu, H., Gao, C., Tao, F., Zhou, Z., Li, K., Li, L., Ma, C., Xu, P. 2014. Systematic metabolic engineering of *Escherichia coli* for high-yield production of fuel bio-chemical 2, 3-butanediol. *Metabolic Engineering*, **23**, 22-33.

Yang, T., Rao, Z., Zhang, X., Lin, Q., Xia, H., Xu, Z., Yang, S. 2011. Production of 2, 3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. *Journal of Basic Microbiology*, **51**(6), 650-658.

Yang, T., Rao, Z., Zhang, X., Xu, M., Xu, Z., Yang, S.-T. 2013. Improved production of 2, 3-butanediol in *Bacillus amyloliquefaciens* by over-expression of glyceraldehyde-3-phosphate dehydrogenase and 2, 3-butanediol dehydrogenase. *PLoS ONE*, **8**(10), e76149.

Ye, L., Kruckeberg, A.L., Berden, J.A., van Dam, K. 1999. Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter. *J. Bacteriol.*, **181**(15), 4673-4675.

Zelle, R.M., de Hulster, E., van Winden, W.A., de Waard, P., Dijkema, C., Winkler, A.A., Geertman, J.-M.A., van Dijken, J.P., Pronk, J.T., van Maris, A.J. 2008. Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Appl. Environ. Microbiol.*, **74**(9), 2766-2777.

Zeng, A.-P., Biebl, H., Deckwer, W.-D. 1991. Production of 2, 3-butanediol in a membrane bioreactor with cell recycle. *Appl. Microbiol. Biotechnol.*, **34**(4), 463-468.

Zeng, A.P., Byun, T.G., Posten, C., Deckwer, W.D. 1994. Use of respiratory quotient as a control parameter for optimum oxygen supply and scale-up of 2, 3-butanediol production under microaerobic conditions. *Biotechnology and Bioengineering*, **44**(9), 1107-1114.

Zhang, G.-C., Kong, I.I., Kim, H., Liu, J.-J., Cate, J.H., Jin, Y.-S. 2014. Construction of a Quadruple Auxotrophic Mutant of an Industrial Polyploid

Saccharomyces cerevisiae Strain by Using RNA-Guided Cas9 Nuclease. *Appl. Environ. Microbiol.*, **80**(24), 7694-7701.

Zhang, G.-C., Liu, J.-J., Ding, W.-T. 2012a. Decreased xylitol formation during xylose fermentation in *Saccharomyces cerevisiae* due to overexpression of water-forming NADH oxidase. *Appl. Environ. Microbiol.*, **78**(4), 1081-1086.

Zhang, L., Yang, Y., Sun, J.a., Shen, Y., Wei, D., Zhu, J., Chu, J. 2010. Microbial production of 2, 3-butanediol by a mutagenized strain of *Serratia marcescens* H30. *Bioresour. Technol.*, **101**(6), 1961-1967.

Zhang, Y.-W., Tiwari, M.K., Gao, H., Dhiman, S.S., Jeya, M., Lee, J.-K. 2012b. Cloning and characterization of a thermostable H_2O-forming NADH oxidase from *Lactobacillus rhamnosus*. *Enzyme and Microbial Technology*, **50**(4), 255-262.

Zhao, X., Shi, F., Zhan, W. 2015. Overexpression of *ZWF1* and *POS5* improves carotenoid biosynthesis in recombinant *Saccharomyces cerevisiae*. *Letters in Applied Microbiology*, **61**(4), 354-360.

Zverlov, V., Berezina, O., Velikodvorskaya, G., Schwarz, W. 2006. Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. *Appl. Microbiol. Biotechnol.*, **71**(5), 587-597.

국문 초록

2,3-Butanediol (2,3-BD)는 화학 산업에서 다양한 적용 분야를 갖는 범용성 화학 물질이다. 본 연구에서, pyruvate decarboxylase (Pdc)를 제거하고, 2,3-BD 생합성 경로를 도입한 재조합 *Saccharomyces cerevisiae* (BD5)를 통하여, 에탄올 생산 없이 2,3-BD 를 생산할 수 있었다. 하지만, Pdc 의 제거로 인한 cofactor 불균형 문제로 많은 양의 글리세롤이 부산물로 생성되었다. *Lactococcus lactis* 유래의 NADH oxidase 를 발현을 통하여, 세포 내 NADH/NAD^+ 비율을 낮추어, glycerol 의 생산은 줄이고 2,3-BD 의 생산을 증가시킬 수 있었다. 또한, Pdc 의 미세한 발현 조절을 통하여 세포 성장 속도 및 2,3-BD 생산성을 크게 향상시켰다. 또한, NADH oxidase 와 미세 조절된 Pdc 를 발현시킨 균주 (BD5_Ctnox)의 포도당을 탄소원으로 하는 유가식 발효를 통하여, 154.3 g/L 의 농도, 1.98 g/L/h 의 생산성, 0.404 g/g 의 수율로 2,3-BD 를 생산할 수 있었다. 글리세롤 생합성 유전자인 glycerol-3-phosphate dehydrogenase (*GPD*)를 위해서 NADH oxidase 의 발현이 반드시 필요했는데, NADH oxidase 는 산소를 전자수용체로 하여 과량 생성된 NADH 를

효율적으로 산화시키기 때문이다. *GPD* 가 제거된 균주의 유가식 배양을 통하여 포도당으로부터 108.6 g/L 의 2,3-BD 를 76 시간만에 생산할 수 있었다. 이 때의 2,3-BD 수율은 0.462 g/g 으로, 이론 수율의 92.4%에 해당하였다.

본 연구에서 구축 된 2,3-BD 생산용 재조합 *S. cerevisiae* 균주는 높은 농도, 수율 및 생산성의 2,3-BD 를 생산할 수 있었다. 특히, 154.3 g/L 의 2,3-BD 는 미생물 발효를 통해 생산된 지금까지 보고된 것 중 가장 높은 값이었다. 본 연구에서 사용한 cofactor engineering 과 pathway engineering 전략은, pyruvate 를 전구체로 하는 다른 화학 물질 합성에도 유용하게 적용될 수 있을 것이다.

주요어 : 2,3-Butanediol (2,3-BD), *Saccharomyces cerevisiae*, NADH oxidase, cofactor engineering, pyruvate decarboxylase, 유가식 배양

Appendix 1. Comparison of intracellular metabolites of BD5_G1CtPDC1 at aerobic condition (N0) and BD5_Ctnox strains at microaerobic condition (N50).

Metabolites	Means		t-value	p	Standard deviation		F-ratio Variances	p Variances
	N50	N100			N50	N100		
3-phosphoglyceric acid	482.9	1623	-24.6771	0.000000	58.0	85.5	2.17	0.470631
aminomalonate	669.2	1635	-19.8597	0.000000	60.9	90.1	2.19	0.467423
acetolactate	2378.2	5810	-17.0846	0.000000	184.4	409.5	4.93	0.151499
glycine	138312.9	330871	-15.8813	0.000000	10891.4	24828.1	5.20	0.139449
succinate	78448.6	175259	-15.4091	0.000000	5228.4	13039.3	6.22	0.104479
5'-deoxy-5'-methylthioadenosine	15471.2	25815	-10.5238	0.000006	1283.3	1784.2	1.93	0.538985
2-(4-hydroxyphenyl)ethanol	973.9	1543	-9.6300	0.000011	94.7	92.3	1.05	0.961765
cytindine-5'-monophosphate	439.8	645	-6.9830	0.000115	50.7	41.7	1.48	0.715108
adenosine-5-monophosphate	981.2	1755	-6.8126	0.000136	234.4	97.6	5.78	0.117829
asparagine	16981.1	22484	-6.2635	0.000242	1476.7	1295.7	1.30	0.806087
pyrophosphate	4437.0	7128	-6.1220	0.000283	722.8	665.9	1.18	0.877606
galactinol	4079.3	5551	-6.0640	0.000301	397.3	369.6	1.16	0.891925
isoleucine	1450.4	2390	-5.3816	0.000660	360.9	149.2	5.85	0.115307
nicotinamide	2249.6	3027	-4.6262	0.001696	293.4	234.7	1.56	0.675692
N-carbamoylaspartate	53.9	90	-4.0497	0.003686	10.3	17.3	2.83	0.338513
salicylaldehyde	8663.0	10264	-3.5941	0.007043	664.6	741.7	1.25	0.836686

aspartate	6509.5	9276	-3.4488	0.008710	1306.4	1229.0	1.13	0.908565
oxoproline	897864.9	1121325	-3.2239	0.012167	137133.4	72221.2	3.61	0.241936
glycerate	103.7	138	-2.9150	0.019438	21.1	15.8	1.79	0.586314
pelargonic acid	112.0	175	-2.9017	0.019839	11.3	47.5	17.68	0.016581
capric acid	328.1	455	-2.5149	0.036092	29.3	108.5	13.75	0.026345
maltotriose	38.3	213	-2.2240	0.056827	29.1	173.5	35.65	0.004385
3-phenyllactate	57.2	124	-1.9678	0.084629	15.8	74.8	22.45	0.010601
terephtalic acid	58.9	85	-1.8603	0.099881	21.0	23.0	1.20	0.862934
malate	2199.4	2403	-1.7895	0.111325	218.1	130.2	2.81	0.341192
tagatose	8965.8	19036	-1.6640	0.134687	1674.3	13428.2	64.32	0.001392
tryptophan	5272.9	5795	-1.5120	0.168976	625.0	454.1	1.89	0.551146
cholic acid	129.8	156	-1.4479	0.185666	27.7	30.2	1.19	0.870362
fructose-6-phosphate	241.4	442	-1.4306	0.190420	271.8	157.6	2.97	0.316089
fructose	972.7	1480	-1.2387	0.250570	133.5	906.4	46.10	0.002667
lactulose	675.5	1431	-1.1296	0.291377	84.2	1493.0	314.43	0.000060
benzoate	4262.1	4754	-1.0433	0.327309	976.2	399.8	5.96	0.111907
asparagine dehydrated*	709.3	773	-0.8663	0.411573	53.4	154.3	8.35	0.063693
lauric acid	175.7	188	-0.8624	0.413581	25.7	17.8	2.09	0.491699
lignoceric acid	470.1	523	-0.6160	0.554987	112.7	155.5	1.90	0.548605
trehalose-6-phosphate	140.6	140	0.0087	0.993238	140.4	40.7	11.91	0.034117
leucine	1962.1	1796	0.2114	0.837875	187.8	1750.6	86.92	0.000770

xylose	118.5	115	0.2284	0.825092	20.0	25.8	1.66	0.634957
glycerol-α-phosphate	15222.4	13419	0.2632	0.799068	14590.0	4671.0	9.76	0.048645
phenylacetate	103.0	99	0.3529	0.733256	9.0	26.1	8.44	0.062568
melibiose	762.8	726	0.3903	0.706474	130.3	169.2	1.69	0.624602
behenic acid	37.8	33	0.4988	0.631354	10.9	19.6	3.22	0.284171
heptadecanoic acid	165.9	156	0.5133	0.621583	33.1	26.8	1.52	0.692778
cyano-L-alanine	111.9	96	0.6561	0.530156	38.5	39.2	1.03	0.975087
glycerol	606352.4	543603	0.7941	0.450068	165876.9	60891.5	7.42	0.077913
2-hydroxyvalerate	541.5	498	0.8531	0.418429	57.8	99.4	2.95	0.319410
proline	961.5	701	0.8827	0.403159	113.3	649.8	32.90	0.005117
tyrosine	21508.1	18083	1.0014	0.345955	7542.9	1262.2	35.71	0.004371
stearic acid	61458.4	55438	1.0162	0.339292	5064.8	12241.0	5.84	0.115700
arachidic acid	118.1	98	1.0646	0.318133	34.4	25.3	1.84	0.568625
thymine	103.1	81	1.0830	0.310352	33.0	31.8	1.08	0.944009
sedoheptulose-7-p	673.0	590	1.2090	0.261165	137.7	68.0	4.10	0.200821
ribulose-5-phosphate	49.8	44	1.2689	0.240142	6.6	7.3	1.23	0.846246
squalene	107.5	77	1.3997	0.199179	47.6	7.4	41.04	0.003340
inosine 5'-monophosphate	57.2	45	1.4126	0.195472	9.7	16.9	3.05	0.305534
2-hydroxypyridine	1790.5	1667	1.4510	0.184832	130.9	137.8	1.11	0.922909
saccharopine	844.0	775	1.4948	0.173330	72.7	73.1	1.01	0.990647
palmitic acid	57013.5	50072	1.5233	0.166174	4279.7	9246.1	4.67	0.164825

β-hydroxybutyrate	1999.4	1682	1.5852	0.151580	425.7	140.8	9.15	0.054465
myo-inositol	7949.7	7320	1.6384	0.139965	712.9	479.9	2.21	0.462177
phenylpyruvate	590.6	393	1.6874	0.129999	77.8	250.1	10.32	0.044040
1,5-anhydroglucitol	372.6	178	1.7294	0.121998	251.0	9.5	697.46	0.000012
2-ketoadipate	320.2	239	1.7391	0.120213	88.5	55.9	2.51	0.394392
α-ketoglutarate	3289.7	2143	1.7439	0.119339	1341.2	601.5	4.97	0.149471
carnitine	819.0	697	1.7526	0.117773	145.1	57.1	6.46	0.098201
levoglucosan	154.0	77	1.7787	0.113173	84.6	47.0	3.24	0.280859
octadecanol	105.3	77	1.7957	0.110273	30.5	16.8	3.29	0.275748
ribose-5-p	326.2	296	1.8205	0.106167	31.4	19.0	2.72	0.356364
1-monopalmitin	162.5	64	1.9573	0.086020	108.5	30.6	12.53	0.031140
glycolate	4586.9	3102	2.1668	0.062123	1515.8	226.8	44.66	0.002836
spermidine	2316.2	1902	2.1671	0.062100	415.8	100.6	17.07	0.017692
xanthine	193.4	119	2.2110	0.057986	74.5	13.7	29.59	0.006272
pyrrole-2-carboxylate	277.2	243	2.2216	0.057039	31.0	15.2	4.15	0.196960
O-phosphorylethanolamine	276.2	158	2.2222	0.056990	83.0	84.5	1.04	0.971557
N-methylalanine	504.3	336	2.2908	0.051199	163.1	20.9	61.02	0.001543
γ-aminobutyrate	1575.5	1302	2.5450	0.034440	214.1	108.6	3.89	0.216704
fumarate	6371.2	4667	2.5648	0.033396	961.3	1133.1	1.39	0.757737
glucose-6-phosphate	774.9	625	2.6176	0.030763	74.4	104.0	1.96	0.531785
3-hydroxypropionate	224.3	187	2.6754	0.028125	28.9	12.2	5.58	0.124395

cysteine	2568.6	1655	2.7656	0.024463	670.0	311.4	4.63	0.166991
urea	13968.7	6044	2.7951	0.023372	5210.8	3610.5	2.08	0.494776
valine	73698.9	10345	2.8943	0.020067	48871.4	2695.7	328.68	0.000055
isocitrate	145764.3	11112	2.9231	0.019200	103000.5	912.9	12729.01	0.000000
mannose	509630.5	288145	2.9956	0.017185	34836.9	161613.2	21.52	0.011479
L-cysteine	1808.7	1523	3.1748	0.013099	144.8	140.1	1.07	0.950046
lactate	49061.8	40450	3.2021	0.012572	4177.6	4325.2	1.07	0.947982
alanine	12854.7	8603	3.2281	0.012091	2368.1	1750.7	1.83	0.572785
myristic acid	13000.1	9202	3.2473	0.011748	914.1	2450.7	7.19	0.082219
glutamate	168113.4	73426	3.2516	0.011672	59207.6	27099.0	4.77	0.159208
galactonate	172.5	112	3.3964	0.009411	19.1	35.1	3.39	0.264164
putrescine	328.6	255	3.4570	0.008606	43.5	19.4	5.01	0.147482
threonine	45896.8	27009	3.4693	0.008452	3445.4	11676.1	11.48	0.036438
2-ketoisocaproic acid	218.8	140	3.6942	0.006093	46.9	9.3	25.58	0.008278
2-hydroxyhexanoate	220.4	165	3.7035	0.006012	31.4	11.5	7.44	0.077522
ergosterol	78.9	48	3.7692	0.005472	16.0	9.1	3.10	0.299466
uracil	967.2	544	3.8124	0.005145	193.4	155.4	1.55	0.682004
β-alanine	410.2	286	3.8294	0.005022	67.8	26.5	6.55	0.096003
oleic acid	234.9	162	3.9349	0.004327	39.6	12.7	9.69	0.049250
phosphogluconate	183.8	105	3.9881	0.004016	42.9	10.9	15.51	0.021134
phosphate	79847.7	53566	4.3399	0.002479	9916.7	9220.9	1.16	0.891261

lysine	62698.0	40823	4.3402	0.002478	4946.4	10126.4	4.19	0.194060
sedoheptulose	95.3	47	4.7863	0.001379	18.7	13.1	2.03	0.508731
adenosine	1114.4	777	5.1173	0.000910	140.7	44.9	9.81	0.048176
phenylalanine	19756.8	13400	5.5510	0.000540	2424.7	823.5	8.67	0.059745
uridine	184.7	81	5.7067	0.000451	21.3	34.3	2.59	0.378878
inosine	647.7	310	6.0309	0.000312	70.7	103.1	2.13	0.481649
ribose	1815.1	910	6.0320	0.000312	326.0	80.0	16.59	0.018653
arabitol	1938.8	264	6.0364	0.000311	619.2	38.7	256.00	0.000091
serine	47371.7	32033	6.0730	0.000298	5175.5	2260.8	5.24	0.137599
threose	489.3	186	6.1704	0.000268	89.1	64.3	1.92	0.542433
histidine	2858.4	1942	6.6834	0.000155	281.5	121.8	5.34	0.133642
citrate	16246.5	11105	7.2458	0.000088	1391.1	763.3	3.32	0.271754
ornithine	140882.7	97679	7.2804	0.000085	10189.5	8500.0	1.44	0.733877
L-homoserine	1558.5	455	7.3071	0.000083	260.2	215.1	1.46	0.720871
palmitoleic acid	928.5	613	7.7173	0.000057	83.0	38.0	4.78	0.158716
methionine	10708.4	6159	7.7313	0.000056	1044.1	800.7	1.70	0.619580
mannitol	5367.7	2290	8.2306	0.000036	816.1	182.0	20.11	0.013038
pentadecanoic acid	11002.5	2790	8.4609	0.000029	804.2	2016.0	6.28	0.102755
L-citrulline	3692.0	2532	8.5494	0.000027	268.2	141.6	3.59	0.243620
adipate	1359.0	696	8.6023	0.000026	164.9	50.1	10.84	0.040416
glutamine	4298.1	2166	8.6341	0.000025	310.6	456.7	2.16	0.473624

lactose	1087.2	648	8.9356	0.000020	95.4	54.4	3.08	0.301865
butane-2,3-diol	31228.3	4063	9.4681	0.000013	6353.2	891.9	50.74	0.002213
oxalate	1449.1	881	9.7363	0.000010	106.6	75.1	2.01	0.514226
ethanolamine	22272.0	12008	11.1815	0.000004	1943.1	661.4	8.63	0.060224
cellobiose	21803.7	11059	11.4647	0.000003	2002.0	619.1	10.46	0.043044
pyruvate	13711.5	5841	13.8132	0.000001	1232.7	321.9	14.67	0.023401
glucose	409094.5	169731	17.6197	0.000000	28733.8	9855.7	8.50	0.061819
2,3-dihydroxy-isovalerate	3259.1	695	17.7640	0.000000	317.6	57.5	30.54	0.005903