Potential of a *Saccharomyces cerevisiae* recombinant strain lacking ethanol and glycerol biosynthesis pathways in efficient anaerobic bioproduction

Takashi Hirasawa¹, Yoshihiro Ida², Chikara Furusawa³, and Hiroshi Shimizu²,*

¹Department of Bioengineering; Tokyo Institute of Technology; Kanagawa, Japan; ²Department of Bioinformatic Engineering; Graduate School of Information Science and Technology; Osaka University; Osaka, Japan; ³Quantitative Biology Center; RIKEN; Osaka, Japan

*Correspondence to: Hiroshi Shimizu; Email: shimizu@ist.osaka-u.ac.jp
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*Saccharomyces cerevisiae* shows high growth activity under low pH conditions and can be used for producing acidic chemicals such as organic acids as well as fuel ethanol. However, ethanol can also be a problematic by-product in the production of chemicals except for ethanol. We have reported that a stable low-ethanol production phenotype was achieved by disrupting 6 NADH-dependent alcohol dehydrogenase genes of *S. cerevisiae*. Moreover, the genes encoding the NADH-dependent glycerol biosynthesis enzymes were further disrupted because the ADH-disrupted recombinant strain showed high glycerol production to maintain intracellular redox balance. The recombinant strain incapable producing ethanol and glycerol could have the potential to be a host for producing metabolite(s) whose biosynthesis is coupled with NADH oxidation. Indeed, we successfully achieved almost 100% yield for L-lactate production using this recombinant strain as a host. In addition, the potential of our constructed recombinant strain for efficient bioproduction, particularly under anaerobic conditions, is also discussed.

**Introduction**

The yeast *Saccharomyces cerevisiae* has been widely used in the production of alcoholic beverages and the fuel ethanol because of its high ethanol production ability. Moreover, since *S. cerevisiae* exhibits high growth activity under low pH conditions, it has been utilized for the production of acidic chemicals such as lactate and succinate. When producing useful chemicals, the production of ethanol as a by-product by *S. cerevisiae* can be problematic. Indeed, metabolic engineering approaches of *S. cerevisiae* have been reported for reducing ethanol production and enhancing the productivity of target metabolite(s).

In cellular metabolism, redox balance (i.e., intracellular balance between NAD(P)H and NAD(P)+) is maintained. Under aerobic conditions, NADH produced via glycolysis and the TCA cycle is oxidized in the respiratory chain. On the other hand, under anaerobic conditions, NADH produced via glycolysis is oxidized in fermentation pathways, such as ethanol and lactate production pathways. For *S. cerevisiae*, NADH produced via glyceraldehyde-3-phosphate dehydrogenase is mainly oxidized via the ethanol biosynthesis catalyzing alcohol dehydrogenases (ADHs). In addition, other metabolic reactions such as glycerol biosynthesis pathways are also involved in NADH oxidation. Since the intracellular redox imbalance can be problematic for bioproduction, it is very important to consider the intracellular redox balance in metabolic engineering.

Recently, we examined the characteristics of an ADH1-disrupted *S. cerevisiae* strain in continuous culture under anaerobic conditions. However, complete loss of ethanol production ability in this recombinant strain could not be achieved, and glycerol production...
was enhanced under anaerobic conditions. Therefore, all NADH-dependent ethanol biosynthesis pathways and glycerol biosynthesis pathways were disrupted in *S. cerevisiae.* The utility of this *S. cerevisiae* recombinant strain that was incapable of both ethanol and glycerol biosynthesis for bioproduction under anaerobic conditions was further examined.

In this paper, maintenance of the intracellular redox balance between NADH and NADPH in *S. cerevisiae* is summarized. In addition, characteristics of *S. cerevisiae* recombinant strains that do not produce ethanol and glycerol and the potential of such recombinant strains for bioproduction under anaerobic conditions are discussed.

**Figure 1.** Central carbon metabolism of *S. cerevisiae*. The oxidation and reduction of NADH in glycolysis and ethanol and glycerol biosynthesis reactions are shown. Glc, glucose; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; Pyr, pyruvate; AcAld, acetaldehyde; G3P, glycerol-3-phosphate.

**Characteristics of *S. cerevisiae* Recombinant Strains Incapable of Ethanol Biosynthesis under Anaerobic Conditions**

As described above, since ethanol becomes a by-product in bioproduction processes other than ethanol production, reduction of ethanol production is important for such bioproduction, particularly anaerobic bioproduction. To that end, we analyzed the *ADH1*-disrupted strain of *S. cerevisiae* and to the cytoplasm, and Adh3p and Adh4p are localized to mitochondria. The Sfa1p protein is annotated as a bifunctional enzyme ADH/S-(hydroxymethyl) glutathione dehydrogenase.

In *S. cerevisiae*, the metabolic flux of glyceraldehyde-3-phosphate dehydrogenase in glycolysis, wherein NADH is reduced, is larger than that of ADHs coupled with NADH oxidation, because of the high carbon requirement for anabolic metabolism (i.e., biomass production) such as amino acid and fatty acid biosynthesis. Therefore, other NADH oxidation reactions are necessary to maintain the NADH/NAD⁺ balance. It has been reported that glycerol production was enhanced by disrupting the *ADH1* gene. In addition, disrupting glycerol biosynthesis reactions enhanced ethanol production. Therefore, glycerol biosynthesis reactions also contribute to maintaining the NADH/NAD⁺ balance. In glycerol biosynthesis reactions, glycerol-3-phosphate dehydrogenases encoding *GPD1* and *GPD2* convert dihydroxyacetone phosphate to glycerol-3-phosphate and require NADH as a reducing power. Metabolic flux analysis revealed that 8.5% of incorporated carbons flowed into glycerol production.

Other NADH oxidation mechanisms include respiration of cytosolic NADH by external mitochondrial NADH dehydrogenase and the glycerol-3-phosphate shunt and NADH oxidation by mitochondrial NADH dehydrogenase in the respiratory chain. Moreover, shuttle mechanisms such as the malate-oxaloacetate shuttle, malate-aspartate shuttle and malate-pyruvate shuttle have also been proposed.

In metabolic networks, many oxidation and reduction reactions are included. For oxidation and reduction reactions, reducing powers such as NADH and NADPH and the reduced forms NAD⁺ and NADPH⁺, respectively, are used, and the intracellular balances for such reducing powers (i.e., NADH/NAD⁺ and NADPH/NADPH⁺ ratios) have to be maintained. In most microorganisms, NADH is produced in the glycolysis and the TCA cycle and is oxidized to NAD⁺ by the respiratory chain under aerobic conditions. For *S. cerevisiae*, NADH produced in glycolysis is dominantly oxidized by ethanol fermentation pathways catalyzed by NADH-dependent ADHs to maintain the redox balance (Fig. 1). Metabolic flux analysis revealed that 74% of carbons incorporated in the cells as glucose flowed to ethanol biosynthesis. *S. cerevisiae* possesses 6 NADH-dependent ADHs encoding *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5* and *SFA1*. Among these, Adh1p is a 150-kDa protein and is the major ADH for ethanol production. Adh2p is thought to be utilized for ethanol assimilation. Adh1p, Adh2p and Adh5p are localized
examined the culture characteristics of the constructed strain. As expected, ethanol yield in the ADH1-disrupted strain was lower than that in the parental strain. Moreover, glycerol yield in the ADH1-disrupted strain was higher than that in the parent strain, suggesting that oxidation of NADH produced by glycolysis was oxidized by glycerol biosynthesis reactions, as a substitute to ethanol biosynthesis pathways.

It is noteworthy that the ADH1-disrupted strain showed unstable phenotypes in continuous culture under oxygen-limited conditions. The ethanol production levels became low at the initial steady-state by 100 h. However, ethanol production levels gradually increased after reaching the initial steady-state. The ethanol production yield was increased, while the glycerol production yield was decreased, indicating that NADH produced via glycolysis was oxidized by the remaining ethanol biosynthesis reactions in the ADH1-disrupted strain under oxygen-limited conditions rather than by glycerol biosynthesis reactions. Moreover, expression of the genes encoding ADH isozymes in the ADH1-disrupted strain, particularly ADH2 and ADH4, were upregulated after long-term continuous culture, probably to compensate for the requirement of oxidation of NADH produced via glycolysis. Therefore, the recombinant strain in which the 6 NADH-dependent ADH isozyme genes (i.e., ADH1, ADH2, ADH3, ADH4, ADH5 and SFA1) were disrupted was constructed and examined under continuous culture.

Ethanol production levels became low throughout the cultivation, but glycerol production levels became high, suggesting that glycerol biosynthesis reactions were utilized for NADH oxidation in the recombinant strain in which the 6 NADH-dependent ADH genes were disrupted. The low ethanol production phenotype is stably shown when all the NADH-dependent ADH genes in S. cerevisiae are disrupted.

Since the recombinant strain incapable of ethanol and glycerol biosynthesis does not possess the 2 major NADH oxidation pathways, it can be expected that production of the target product whose biosynthesis is coupled with NADH oxidation can be achieved by a metabolic engineering approach. We introduced the gene for human L-lactate dehydrogenase (LDH) into the recombinant strain incapable of ethanol and glycerol biosynthesis and examined L-lactate production under anaerobic conditions, which was achieved by N2 gas flow into the jar bioreactor. LDH converts 1 mol pyruvate to 1 mol L-lactate; this conversion is coupled with oxidation of 1 mol NADH. If the theoretical maximum yield is achieved, 2 mol L-lactate is produced from 1 mol glucose, and 2 mol NADH produced by the glyceraldehyde-3-phosphate dehydrogenase reaction are oxidized, which is coupled with the LDH reaction, to maintain redox balance. In addition, since the Michaelis-Menten constant for human LDH is lower than those of some lactic acid bacteria, we selected human LDH for constructing L-lactate-producing recombinant strain.

The recombinant strain incapable of both ethanol and glycerol biosynthesis could not grow under anaerobic conditions probably due to the NADH accumulation. Therefore, cells of the recombinant strain cultivated aerobically were suspended in fresh medium at a high density and packed in the jar bioreactor. Then, L-lactate production was examined by incubating the packed cells with gentle

Figure 2. L-Lactate production by the human LDH-carrying S. cerevisiae recombinant strain with the 6 NADH-dependent ADH isozyme genes. Cells of the recombinant strains carrying empty plasmid (A) and the human-LDH-expression plasmid (B) were incubated in the jar bioreactor with a N2 gas purge and slow agitation. Cell growth (OD660, circles) and concentrations of glucose (squares), ethanol (diamonds) and L-lactate (triangles) are shown.
agitation and a N₂ gas purge (Fig. 2). Although no cell growth was observed as expected, glucose was consumed and L-lactate was produced. Moreover, almost all glucose incorporated into the cells was efficiently converted to L-lactate; i.e., the mol-based L-lactate yield was around 200%. The efficient L-lactate production process under anaerobic conditions could be established by using the *S. cerevisiae* recombinant strain incapable of ethanol and glycerol biosynthesis.

### Potential of the *S. cerevisiae* Recombinant Strain Incapable of Ethanol and Glycerol Biosynthesis

The *S. cerevisiae* recombinant strain incapable of ethanol and glycerol biosynthesis could be used for anaerobic bioproduction. In particular, the metabolite(s) whose biosynthesis is coupled with NADH oxidation such as L-lactate could be suitable targets for anaerobic bioproduction using this recombinant strain.

Recently, in silico metabolic simulation using genome-scale models have been utilized in metabolic engineering. In this simulation, metabolic flux distribution at a steady-state can be simulated as the biomass formation is maximized. This simulation platform can predict metabolic state changes against culture environments and gene knockouts for improvement of target metabolite production. Therefore, in silico metabolic simulation using a genome-scale metabolic model of *S. cerevisiae* iND750 was applied to search for target metabolites for anaerobic bioproduction by the recombinant strain incapable of ethanol and glycerol biosynthesis. The feasibility of production of selected metabolite(s) whose biosynthesis is alternative to that of ethanol and glycerol in terms of NADH oxidation under anaerobic conditions can be surveyed by our in silico simulation studies. As a result, we found 20 native *S. cerevisiae* metabolites whose biosynthesis reactions involved oxidation of NADH as an alternative to ethanol and glycerol biosynthesis. The feasibility of production of the identified 20 metabolites, except for the target metabolite, as well as ethanol and glycerol biosynthesis reactions based on the simulation: alanine, asparagine, aspartate, cysteine, D-glucosamine-6-phosphate, D-sorbitol, DTPP, fumarate, isoleucine, malate, methionine, ornithine, (R)-pantothenate, phenylalanine, proline, succinate, threonine, tryptophan, tyrosine and valine. We further simulated whether each metabolite could be produced under anaerobic conditions, and the only solution where the target could be produced without biomass formation was feasible. In this simulation, the fluxes for biosynthesis of the identified 20 metabolites, except for the target metabolite, as well as ethanol and glycerol biosynthesis were set to zero in advance. Among the 20 metabolites, alanine, valine and succinate were produced without biomass formation under anaerobic conditions; feasible solutions on the production of target metabolites without biomass formation could be estimated. 

Figure 3 shows the simulated flux distributions when producing alanine, valine and succinate as well as L-lactate, whose production requires heterologous metabolic pathway, under completely anaerobic conditions. In all cases, glucose incorporated into the cells was completely converted to pyruvate, and the converted pyruvate was further completely converted to the target metabolites. Moreover, there was no biomass formation; i.e., achieving the theoretical maximum yield and maintenance of intracellular redox balance was simulated.

Based on the concepts of synthetic biology, introduction of heterologous metabolic pathway(s) into the host species is effective for producing non-native metabolite(s). If the biosynthesis pathways for the target metabolite involve
NADH-coupled reactions, utilization of our S. cerevisiae recombinant strain with elimination of both ethanol and glycerol biosynthesis as a host would be effective as shown above for the case of L-lactate production (Fig. 2). We recently established a platform to design heterologous metabolic pathways to produce metabolite(s) that cannot be produced by the host.23 By using our simulation platform, the design of the metabolic pathways and bioproduction are facilitated. Moreover, similar to the L-lactate production described above, by constructing the recombinant strain producing the target metabolite(s) based on the strain incapable of ethanol and glycerol biosynthesis, efficient anaerobic production processes with nearly the theoretical maximum yield might be able to be established. However, further studies are required.

We constructed the S. cerevisiae recombinant strain incapable of both ethanol and glycerol biosynthesis for bioproduction. As shown in our report on L-lactate production, high yields of target product can be achieved using this recombinant strain as a host. However, glucose consumption and target product production rates were very low. Moreover, the cell growth rate under aerobic conditions was also low in comparison with the parent strain. In our recombinant strain, glycerol biosynthesis is impaired due to the disruption of GPDI and GPD2 genes. In the GPDI and GPD2 knockout strain of S. cerevisiae, one of the important metabolites for phospholipid biosynthesis, glycerol-3-phosphate, which is a precursor for phosphatidic acid biosynthesis, cannot be produced from dihydroxyacetone phosphate directly. However, S. cerevisiae possess the bypass reactions for glycerol-3-phosphate biosynthesis; glycerol-3-phosphate is produced from 1-acyl-dihydroxyacetone phosphate which is produced by acylation of dihydroxyacetone phosphate.24-25 Thus, the GP DI and GPD2 knockout strain is viable without nutrient supplementation. Additionally, the GP DI gene encoding pyruvate decarboxylase involved in acetaldehyde biosynthesis, which is necessary for cytosolic acetyl-CoA biosynthesis, is also disrupted in our recombinant strain. However, since S. cerevisiae possesses 3 genes encoding pyruvate decarboxylase isozymes (PDC1, PDC5 and PDC6),26-28 our recombinant strain can produce acetaldehyde by the Pdc5p and Pdc6p proteins. Therefore, the reason why our recombinant strain shows low cell growth, glucose consumption and target product production rates of our recombinant strain is caused by disruption of the major metabolic reactions involved in NADH oxidation (i.e., ethanol and glycerol biosynthesis pathways) rather than the insufficiency of nutrients.

For efficient bioproduction, it is necessary to improve these parameters for the recombinant strain. We will continue to study our constructed recombinant strain for efficient bioproduction by S. cerevisiae.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

References
1. Cordier H, Mendes F, Vasconcelos I, François JM. A metabolic and genomic study of engineered Saccharomyces cerevisiae strains for high glycerol production. Metab Eng 2007; 9:364-78; PMID:17500021; http://dx.doi.org/10.1016/j.ymben.2007.03.002
2. van Maris AJ, Winkler AA, Porro D, van Dijken JP, Pronk JT. Homofermentative lactic acid production cannot sustain anaerobic growth of engineered Saccharomyces cerevisiae: possible consequence of energy-dependent lactate export. Appl Environ Microbiol 2004; 70:2898-905; PMID:15128549; http://dx.doi.org/10.1128/AEM.70.5.2898-2905.2004
3. Takahiro K, Ishida N, Nagamori E, Saitoh S, Onishi T, Kondo A, Takahashi H. Double mutation of the ADH1 and ADHI genes improves lactate production in the yeast Saccharomyces cerevisiae expressing the bovine lactate dehydrogenase gene. Appl Microbiol Biotechnol 2009; 82:883-90; PMID:19122995; http://dx.doi.org/10.1007/s00253-008-1833-5
4. Okano K, Tanaka T, Ogino C, Fukuda H, Kondo A. Biotechnological production of enantiomerically pure lactic acid from renewable resources: recent achievements, perspectives, and limits. Appl Microbiol Biotechnol 2009; 82:427-32; http://dx.doi.org/10.1007/s00253-009-1772-0
5. Bakker BM, Overkamp KM, Kötter P, Luttik MA, Pronk JT, van Dijken JP, et al.; van Maris AJ; van Dijken JP. Stoichiometry and compartmentation of NAD metabolism in Saccharomyces cerevisiae. FEMS Microbiol Rev 2001; 25:15-37; PMID:11512939; http://dx.doi.org/10.1111/j.1574-6976.2001. tb00570.x
6. Ida Y, Furusawa C, Hirasa T, Shimizu H. Stable disruption of ethanol production by deletion of the genes encoding alcohol dehydrogenase isozymes in Saccharomyces cerevisiae. J Biosci Bioeng 2012; 113:192-5; PMID:22030367; http://dx.doi.org/10.1016/j.jbiosc.2011.09.019
7. Ida Y, Hirasa T, Furusawa C, Shimizu H. Utilization of Saccharomyces cerevisiae recombinant strain incapable of both ethanol and glycerol biosynthesis for anaerobic bioproduction. Appl Microbiol Biotechnol 2013; 97:4811-9; PMID:23435983; http://dx.doi.org/10.1007/s00253-013-4760-x
8. Jouhten P, Rintala E, Huusokonen A, Tamminen A, Toivari M, Wibe M, Ruosalon L, Penttilä M, Maahimo H. Oxygen dependence of metabolic fluxes and energy generation of Saccharomyces cerevisiae CEN.PK113-1A. BMC Syst Biol 2008; 2:60; PMID:18613954; http://dx.doi.org/10.1186/1753-0000-2-60
9. de Smidt O, du Preez JC, Albertyn J. The alcohol dehydrogenases of Saccharomyces cerevisiae: a comprehensive review. FEMS Yeast Res 2008; 8:967-78; PMID:18479436; http://dx.doi.org/10.1111/j.1567-1364.2008.00387.x
10. Guadalupe Medina V, Almering MJ, van Maris AJ, Pronk JT. Elimination of glycerol production in anaerobic cultures of a Saccharomyces cerevisiae strain engineered to use acetic acid as an electron acceptor. Appl Environ Microbiol 2010; 76:190-5; PMID:19915031; http://dx.doi.org/10.1128/AEM.01772-09
11. Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L. The two isoenzymes for yeast NAD+-dependent glycerol-3-phosphate dehydrogenase encoded by GP D1 and GP D2 have distinct roles in osmoadaptation and redox regulation. EMBO J 1997; 16:2179-87; PMID:9171333; http://dx.doi.org/10.1093/emboj/16.15.2179
12. Pettit SM, Nealon DA, Henderson AR. Purification of lactate dehydrogenase, isozyme-5 from human liver. Clin Chem 1981; 27:88-93; PMID:7449128
13. Garvie EL. Bacterial lactate dehydrogenases. Microbiol Rev 1980; 44:106-39; PMID:697721
14. LeVan KM, Goldberg E. Properties of human testis-specific lactate dehydrogenase expressed from Escherichia coli. Biochem J 1991; 273:387-92; PMID:1996697
15. Blazek J, Alper H. Systems metabolic engineering: genome-scale models and beyond. Biotechnol J 2010; 5:667-59; PMID:20154446; http://dx.doi.org/10.1002/biot.200900247
16. Oberhardt MA, Palsson BO, Papin JA. Applications of genome-scale metabolic reconstructions. Mol Syst Biol 2009; 5:320; PMID:19888215; http://dx.doi.org/10.1038/msb.2009.77
17. Park JM, Kim TY, Lee SY. Constraints-based genome-scale metabolic simulation for systems metabolic engineering, Biotechnol Adv 2009; 27:979-88; PMID:19464354; http://dx.doi.org/10.1016/j.biotechadv.2009.05.019
18. Duarte NC, Herregård MJ, Palsson BO. Reconstruction and validation of Saccharomyces cerevisiae iND750, a fully compartmentalized genome-scale metabolic model. Genome Res 2004; 14:1298-309; PMID:15597565; http://dx.doi.org/10.1101/gr.225904
19. Lee JW, Na D, Park JM, Lee J. Choi S, Lee SY. Systems metabolic engineering of microorganisms for natural and non-natural chemicals. Nat Chem Biol 2012; 8:536-66; PMID:22596205; http://dx.doi.org/10.1038/nchembio.790
20. Yadav VG, De Mey M, Lim CG, Ajikumar PK, Stephanopoulos G. The future of metabolic engineering and synthetic biology: towards a systematic practice. Metab Eng 2012; 14:233-41; PMID:22609571; http://dx.doi.org/10.1016/j.ymben.2012.02.001
21. Chaturachai S, Furusawa C, Shimizu H. ArtPathDesign: Rational heterologous pathway design system for the production of nonnative metabolites. J Biosci Bioeng 2013; 116:524-7; PMID:23664926; http://dx.doi.org/10.1016/j.jbiosc.2013.04.002
22. Arthenstraedt K, Weys S, Palrauf F, Daum G. Redundant systems of phosphatidic acid biosynthesis via acylation of glycerol-3-phosphate or dihydroxyacetone phosphate in the yeast Saccharomyces cerevisiae. J Bacteriol 1999; 181:4588-63; PMID:10049376

www.landesbioscience.com
23. Racenis PV, Lai JL, Das AK, Mullick PC, Hajra AK, Greenberg ML. The acyl dihydroxyacetone phosphate pathway enzymes for glycerolipid biosynthesis are present in the yeast Saccharomyces cerevisiae. J Bacteriol 1992; 174:5702-10; PMID:1512203

24. Tillman TS, Bell RM. Mutants of Saccharomyces cerevisiae defective in sn-glycerol-3-phosphate acyltransferase. Simultaneous loss of dihydroxyacetone phosphate acyltransferase indicates a common gene. J Biol Chem 1986; 261:9144-9; PMID:3522586

25. Zheng Z, Zou J. The initial step of the glycerolipid pathway: identification of glycerol 3-phosphate/dihydroxyacetone phosphate dual substrate acyltransferases in Saccharomyces cerevisiae. J Biol Chem 2001; 276:41710-6; PMID:11544256; http://dx.doi.org/10.1074/jbc.M104749200

26. Seeboth PG, Bohnsack K, Hollenberg CP. pdc1(0) mutants of Saccharomyces cerevisiae give evidence for an additional structural PDC gene: cloning of PDC5, a gene homologous to PDC1. J Bacteriol 1990; 172:678-85; PMID:2404950

27. Kellermann E, Seeboth PG, Hollenberg CP. Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (PDC1) from Saccharomyces cerevisiae. Nucleic Acids Res 1986; 14:8963-77; PMID:3537965; http://dx.doi.org/10.1093/nar/14.22.8963

28. Hohmann S. Characterization of PDC6, a third structural gene for pyruvate decarboxylase in Saccharomyces cerevisiae. J Bacteriol 1991; 173:7963-9; PMID:1744053