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ORIGINAL PAPER

Effects of deletion of glycerol-3-phosphate dehydrogenase and glutamate dehydrogenase genes on glycerol and ethanol metabolism in recombinant *Saccharomyces cerevisiae*

Jin-Woo Kim · Young-Wook Chin · Yong-Cheol Park · Jin-Ho Seo

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Abstract Bioethanol is currently used as an alternative fuel for gasoline worldwide. For economic production of bioethanol by *Saccharomyces cerevisiae*, formation of a main by-product, glycerol, should be prevented or minimized in order to reduce a separation cost of ethanol from fermentation broth. In this study, *S. cerevisiae* was engineered to investigate the effects of the sole and double disruption of NADH-dependent glycerol-3-phosphate dehydrogenase 1 (GPD1) and NADPH-requiring glutamate dehydrogenase 1 (GDH1) on the production of glycerol and ethanol from glucose. Even though sole deletion of *GPD1* or *GDH1* reduced glycerol production, double deletion of *GPD1* and *GDH1* resulted in the lowest glycerol concentration of 2.31 g/L, which was 46.4% lower than the wild-type strain. Interestingly, the recombinant *S. cerevisiae ΔGPD1ΔGDH1* strain showed a slight improvement in ethanol yield (0.414 g/g) compared with the wild-type strain (0.406 g/g). Genetic engineering of the glycerol and glutamate metabolic pathways modified NAD(P)H-requiring metabolic pathways and exerted a positive effect on glycerol reduction without affecting ethanol production.

Keywords *Saccharomyces cerevisiae* · Ethanol · Glycerol · Glycerol-3-phosphate dehydrogenase 1 (GPD1) · Glutamate dehydrogenase 1 (GDH1)

J.-W. Kim · Y.-W. Chin · J.-H. Seo (✉)
Department of Agricultural Biotechnology,
Seoul National University, Seoul 151-921, Korea
e-mail: jhseo94@snu.ac.kr

Y.-C. Park (✉)
Department of Advanced Fermentation Fusion Science and Technology, Kookmin University,
Seoul 136-702, Korea
e-mail: ycpark@kookmin.ac.kr

Introduction

Ethanol is a promising biofuel and currently used as an alternative fuel for gasoline [1]. For using ethanol as biofuel, ethanol in fermentation broth is purified to anhydrous ethanol by several separation steps [2]. Until now, many fermentation and separation processes were employed to produce ethanol economically [3–7]. Among them, a continuous fermentation/pervaporation technique has advantages over other techniques because of its process simplicity, reduced toxicity toward fermenting microorganisms, and recovery of a concentrated ethanol stream requiring less distillation capacity and energy consumption [5]. The performance and permeability of ethanol-selective membranes used in the pervaporation process was decreased by by-products in fermentation broth including glycerol and organic acids [8]. Among several by-products in ethanol production by an alcohol yeast, *Saccharomyces cerevisiae*, up to 5% of the total carbon sources were converted into glycerol [9]. Addition of glycerol to an ethanol/water mixture resulted in a decrease in the vapor pressures of both ethanol and water, and hence the total flux decreased with increasing glycerol concentrations [8, 10]. The ethanol flux decreased to 50% after addition of 0.8% (w/w) of glycerol [8]. For effective pervaporation of ethanol from fermentation broth, selective removal of glycerol or development of recombinant yeast strains which produce a low level of glycerol are required [10].

Glycerol is formed under anaerobic conditions in order to balance the ratio of NADH and NAD^+ contents in the cytoplasm [11]. Assimilation of sugars into cell biomass results in the formation of excess NADH [12]. The respiratory chain cannot function in anaerobic conditions, thus the excess NADH produced by the assimilation of sugars into cell biomass should be recycled to NAD^+ at a

substrate level. The excess NADH is oxidized through the formation of glycerol at the cost of 1 mol NADH per 1 mol glycerol [13]. Therefore, the formation of glycerol could be decreased by the consumption of excess NADH by alternative metabolic pathways [14].

Glycerol production in yeast is catalyzed by NADH-dependent glycerol 3-phosphate dehydrogenase encoded by the *GPD* gene and glycerol 3-phosphate phosphatase encoded by the *GPP1* gene. *S. cerevisiae* is known to possess two GPD isozymes, GPD1 and GPD2. It was reported that deletion of the *GPD1* or *GPD2* gene led to a decrease in glycerol yield [13, 15–17]. However, surplus formation of NADH resulted in a decrease in the specific growth rate and ethanol productivity [18]. So, additional metabolic pathway should be modified to recycle the excess NADH which is not oxidized in the glycerol metabolism. As shown in Fig. 1, combination of α-ketoglutarate with ammonium ion is catalyzed by NADPH-dependent glutamate dehydrogenase and its isozyme encoded by the *GDH1* and *GDH3* genes in *S. cerevisiae*, respectively [19, 20]. Another reaction for glutamate synthesis in *S. cerevisiae* is catalyzed by NADH-dependent glutamate synthase (GLT1) [21]. Deletion of the *GDH1* gene could block the NADPH-consuming assimilation of ammonium ion resulting in triggering the NADH-oxidizing reaction of glutamate synthesis mediated by GLT1. This scheme gave the reduction of glycerol from glucose [22, 23].

The glycerol and glutamate pathways in *S. cerevisiae* were individually modified in an effort to decrease glycerol. In this study, *S. cerevisiae* strains were engineered to investigate the combined effects of the double deletion of the *GPD1* and *GDH1* genes on production of glycerol and ethanol. Anaerobic batch fermentations and the analysis of related enzymes were carried out to evaluate the synergistic effects of the single and double gene deletion on glycerol production.

Materials and methods

Strains and culture conditions

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was cultivated in LB medium and its transformants were selected as described previously [24]. *S. cerevisiae* CEN.PK2-1D (MATa, *ura3-52*; *trp1-289*; *leu2-3_112*; *his3 D1*; MAL2-*8C*; *SUC2*) was used as a host for ethanol fermentation. YNB medium [6.7 g/L yeast nitrogen base without amino acids (Sigma, USA)] containing 1.92 g/L yeast synthetic drop-out medium without uracil (Sigma, USA) and 20 g/L glucose was used to select *S. cerevisiae*

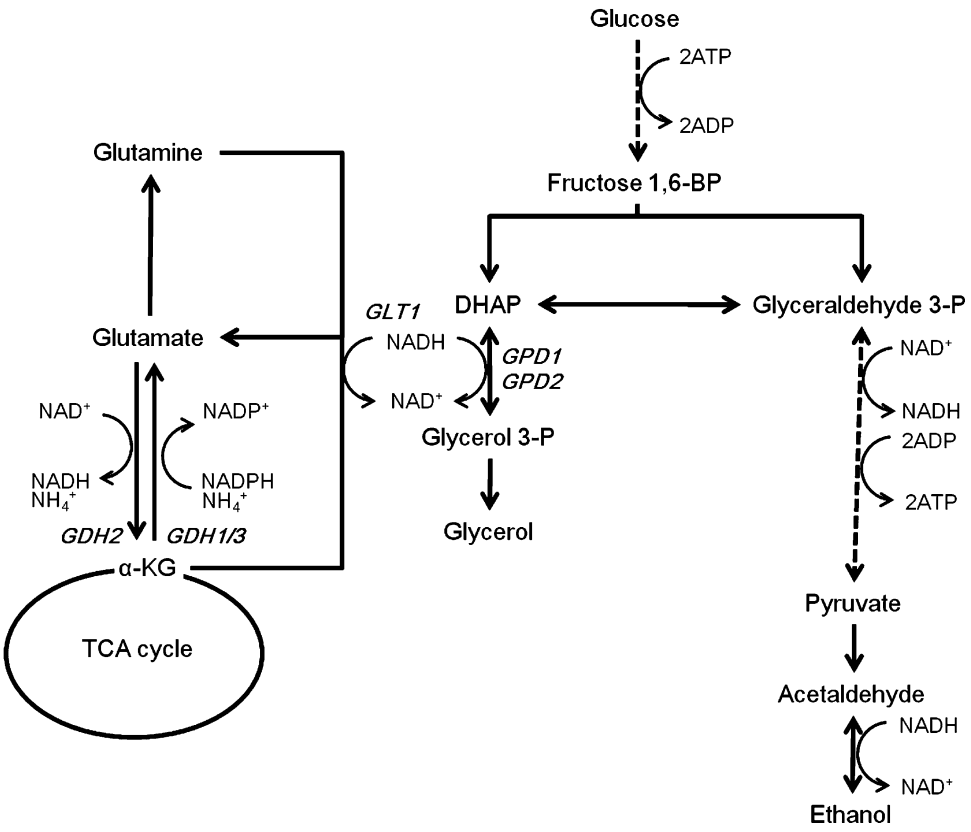


Fig. 1 Metabolic pathways for ethanol and glycerol production, and ammonium assimilation in *S. cerevisiae*. Some metabolites and italicized metabolic enzymes were abbreviated as follows: DHAP, dihydroxyacetone phosphate; fructose 1,6-BP, fructose 1,6-bisphosphate; glyceraldehyde 3-P, glyceraldehydes 3-phosphate; glycerol 3-P, glycerol 3-phosphate; α-KG, α-ketoglutarate; GPD 1 and GPD2, NADH-dependent glycerol 3-phosphate dehydrogenase; GDH1, NADPH-dependent glutamate dehydrogenase; GDH2, NAD^+-dependent glutamate dehydrogenase; GLT1, NADH-dependent glutamine synthase

transformants with the *URA3* gene. Aureobasidin A (0.5 μg/mL, Takara, Japan) was added to select *S. cerevisiae* transformants with the aureobasidin A resistant gene. Anaerobic batch fermentation was carried out in a 1.0 L bioreactor (BIOSTAT-Q, Germany) containing 0.8 L YNB medium with 117 g/L glucose and 1.92 g/L of amino acid mixture (SSP, USA). The culture was performed at 30 °C, pH 5.0 and 500 rpm without aeration and repeated in duplicate.

Genetic manipulation

Two plasmids were constructed to harbor the truncated *GPD1* and *GDH1* genes individually. The *GPD1* and *GDH1* gene fragments were PCR-amplified from the genomic DNA of *S. cerevisiae* CEN.PK2-1D using the primer sets: 5′-ACATGCATGCCATCGTTTTCAACATTCCACATCA-3′ and 5′-GGGGTACCAGTCTTCGACAGAGCCACATGTTT-3′ for the *GPD1* gene fragment, 5′-CCCAAGCTTCTCTACTCTTTTCGAACAACACCC-3′ and 5′-CTAGCTAGCACGCGCTTACCTTCGAAAGATTCC-3′ for the *GDH1* gene fragment. The recognition sites of DNA restriction enzymes were underlined in the primer sequences. The truncated *GPD1* fragments were ligated to the *Sph*I–*Kpn*I site of pAUR101 vector to construct plasmid pAUR101_d_GPD1. The truncated *GDH1* fragments were combined with plasmid pYip5 at the restriction site of *Hin*dIII–*Nhe*I to obtain plasmid pYip5_d_GDH1. The chromosomal *GPD1* and *GDH1* genes were destroyed by a one-step gene disruption strategy. The linearized plasmids of pAUR101_d_gpd1 and pYip5_d_gdh1 by the *Xba*I and *Bgl*II treatment, respectively, were introduced into *S. cerevisiae* CEN.PK2-1D. Yeast transformation was performed by the lithium acetate method as described [24].

Assay

Optical density was measured with a spectrophotometer (Shimadzu, Tokyo, Japan) at 600 nm and dry cell mass was calculated using a pre-determined factor of 0.23 g/L/optical density. Glucose, glycerol and ethanol were analyzed by a high-performance liquid chromatography (1100 series, Agilent, USA) equipped with a Rezex ROA-organic acid column (Phenomenex, USA) and a RI detector (1100 series, Agilent, USA). Column temperature was set at 60 °C and the mobile phase of 5 mM H_2SO_4 was flowed at a rate of 0.6 ml/min.

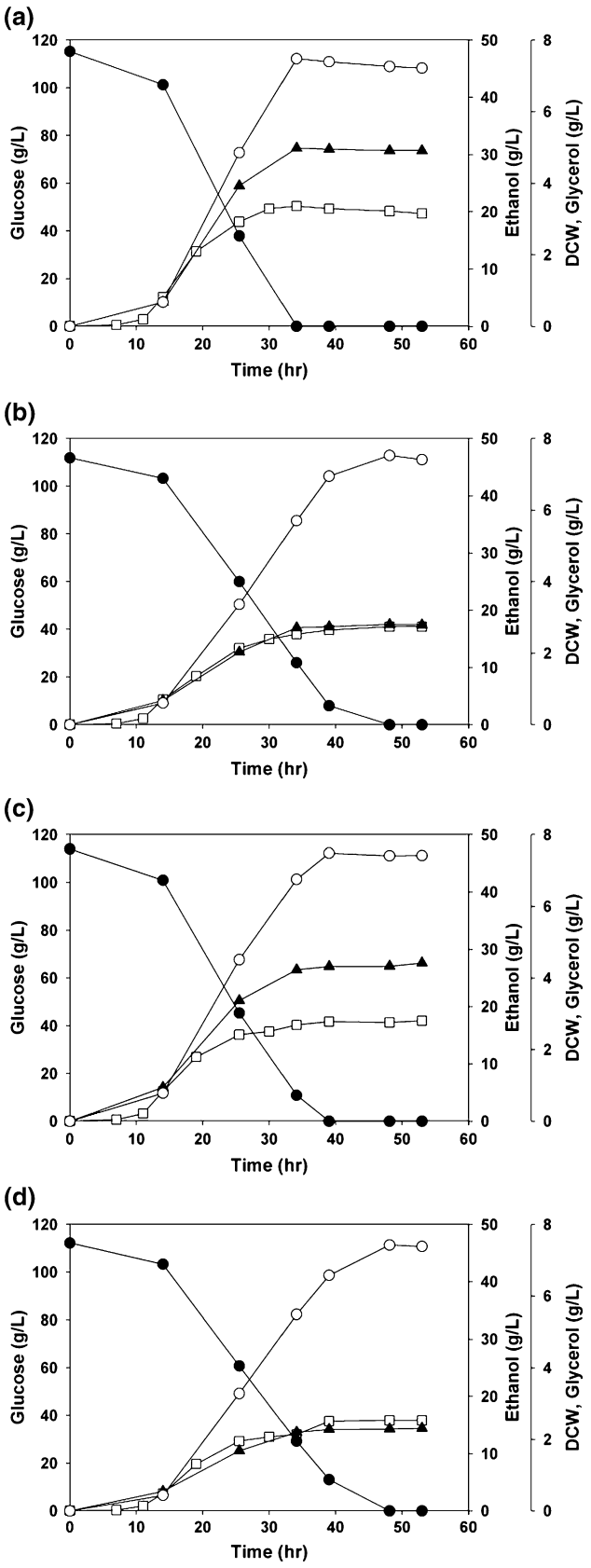
To prepare the crude extract of the yeast cells at the mid-exponential growth phase (19 h), the culture broth was centrifuged and the collected cell pellets were washed twice with double distilled water. The harvested cells were suspended in 100 mM potassium phosphate buffer (pH 7.8), of which optical density was adjusted to 10. After addition of a protease inhibitor solution (Roche, Switzerland) and 1.0 g glass bead (i.d. 0.5 mm, Biospec products, USA) into 1 mL of the cell suspension, the tube was vortexed vigorously for 1 min and kept in ice for 2 min, and this procedure was repeated ten times. After centrifugation for 15 min at 4 °C and 12,000 rpm, the supernatant was used as the crude enzyme solution. Activities of NADPH-dependent glutamate dehydrogenase and NAD^+-dependent glutamate dehydrogenase (GDH2) were assayed as described elsewhere [25]. Determination of glutamate synthase (GLT1) activity followed the previous report [21]. The reduction of NAD^+ and $NADP^+$, and oxidation of NADH were measured by the absorbance changes at 340 nm of wavelength. All enzyme activity assays were performed at 30 °C. One unit of activity was defined as the amount of enzyme reducing (or oxidizing) 1 μmol NAD(P)H per minute at the corresponding reaction conditions. Protein concentration was determined by the Bradford method [24].

Results and discussion

Anaerobic fermentation

Glycerol has been known to be a main by-product in ethanol fermentation by yeasts. It has been reported that reduction of glycerol production could be achieved by deletion of either *GPD1* or *GDH1* [16, 23]. In this study, the effects of the double deletion of both genes were investigated in anaerobic fermentations of recombinant *S. cerevisiae* strains deficient in the chromosomal *GPD1* and/or *GDH1* genes. Profiles of cell growth, glucose consumption and metabolite production were depicted in Fig. 2.

In Fig. 2 and Table 1, deletion of the *GPD1* and *GDH1* genes reduced cell growth rate and final cell mass. Final cell mass of the three *S. cerevisiae* mutants (2.69 ± 0.15 g/L) was 80.0% of that of the wild strain (3.36 g/L). In the previous work, single deletion of the *GPD1* or *GPD2* gene in *S. cerevisiae* gave a similar [17] or slightly decreased growth rate compared with the wild type [13]. *GPD1* and/or *GDH1* deletion also affected glucose consumption, and glycerol and ethanol production. In an aspect of glycerol production, double deletion of *GPD1* and *GDH1* provided the lowest final concentration of glycerol (2.31 g/L), which was 2.2, 1.2 and 1.9 times lower than the values for the wild type and individual disruption of *GPD1* and *GDH1*, respectively (Table 1). Sole deletion of *GPD1* and double deletion of *GPD1* and *GDH1* reduced glucose consumption rate from 3.38 g/L/h (the wild type) to 2.65 ± 0.16 g/L/h. A decrease in glucose consumption rate by deletion of NADH-dependent *GPD1* was resulted from the decline of cell mass which might be ascribed to the reduced ability



◀ **Fig. 2** Anaerobic batch fermentation profiles of *S. cerevisiae* CEN.PK2-1D wild type (**a**), *ΔGPD1* (**b**), *ΔGDH1* (**c**) and *ΔGPD1ΔGDH1* (**d**). *Symbols* are denoted as follows: dry cell weight (DCW, *open square*), glucose (*closed circle*), glycerol (*closed triangle*), ethanol (*open circle*)

of NADH oxidation as reported elsewhere [13]. Along with a decrease in glucose consumption rate, ethanol production rates were reduced by the gene deletion. Maximum ethanol productivities calculated from 14 to 34 h were compared. Sole deletion of *GDH1* and *GPD1*, and the double deletion gave the ethanol productivities of 1.87, 1.59 and 1.58 g/L/h, which were 88, 75 and 74% of the corresponding value for the wild-type strain, respectively. However, the double deletion of *GDH1* and *GPD1* gave a similar ethanol yield (0.414 g/g) to the wild type (0.406 g/g). It is clear that deletion of the *GPD1 and GDH1* genes has a synergetic effect on the reduced production of glycerol and a slight increase or a similar value in ethanol yield compared with the wild-type strain. Similar to this study, it was shown that sole deletion of *GPD1* reduced glycerol concentration and hence elevated ethanol yield by around 4% [16]. In the case of *GDH1* disruption, an industrial strain of *S. cerevisiae* TN1 was engineered to make a *GDH1*-deleted TN9 stain and anaerobic batch fermentation using 25 g/L glucose was carried out [23]. Contrary to our results of marginal changes by *GDH1* deletion, glycerol concentration decreased from 2.6 to 1.3 g/L while the ethanol yield increased by 8% compared with the control TN1 [23]. The reason for these different changes by *GDH1* deletion is unclear. But the genetic and evolutionary backgrounds may be different between the *S. cerevisiae* strains of industrial TN1 and laboratorial CEN.PK2-1D used in this study. More modulation of the ammonium assimilation pathway catalyzed by GLN1 and GLT1 did not improve ethanol production significantly [23], indicating that the ammonium assimilation metabolism is controlled tightly by other factors including ATP and NADPH levels. It is noteworthy that a decrease in glycerol production as a result of double deletion of the *GPD1* and *GDH1* genes did not lead to a significant increase in ethanol production, rather it could be reasonable to say that a metabolic flux to glycerol production was independent of the flux to ethanol production in the *GPD1* and *GDH1* double deletion strain.

Effects of gene deletion on glutamate metabolic enzymes

The *GPD1* and *GDH1* genes modulated in this study encode the metabolic enzymes requiring NADH and NADPH, respectively. Deletion of the two genes in *S. cerevisiae* clearly reduced glycerol production by modifying the NAD(P)H metabolism. A change in NAD(P)H-related

Table 1 Summarized results of batch fermentations of *S. cerevisiae* CEN.PK2-1D wild-type strain and genetically knock-out strains deficient in *GPD1* and/or *GDH1* using 117 g/L glucose as a carbon source

Genotype	Dry cell weight (g/L)	Specific growth rate (h^{-1})	Ethanol yield (g/g)	Ethanol concentration (g/L)	Glycerol concentration (g/L)	Ethanol productivity (g/L/h)
Wild type	3.36	0.473	0.406	46.7	4.98	1.37
ΔGPD1	2.74	0.472	0.420	47.0	2.80	0.98
ΔGDH1	2.81	0.449	0.410	46.8	4.42	1.20
ΔGPD1ΔGDH1	2.53	0.440	0.414	46.4	2.31	0.96

The standard deviations of the results were around 3–4%

Table 2 Specific activities of NADPH-dependent glutamate dehydrogenase, NADH-dependent glutamate dehydrogenase and NADH-dependent glutamate synthase in recombinant *S. cerevisiae* CEN.PK2-1D strains cultivated in anaerobic conditions

Genotype	Enzyme activity(U/mg protein) ± SD		
	NADPH-dependent glutamate dehydrogenase	NAD^+-dependent glutamate dehydrogenase	NADH-dependent glutamate synthase
Wild type	43.0 ± 0.5	14.5 ± 2.0	19.0 ± 0.5
ΔGPD1	38.6 ± 6.2	11.1 ± 0.4	18.4 ± 0.3
ΔGDH1	10.6 ± 2.1	7.7 ± 0.6	25.0 ± 1.3
ΔGPD1GDH1	9.8 ± 1.4	5.5 ± 0.3	21.4 ± 0.3

One unit of activity was defined as the amount of enzyme reducing (or oxidizing) 1 μmol NAD(P)H per minute under the corresponding reaction conditions

enzyme activity was measured to possibly explain why double deletion of the above two genes reduced glycerol production. The crude enzyme extracts were subjected to activity assay of cofactor-requiring glutamate metabolic enzymes (Table 2). In the *S. cerevisiae* CEN.PK2-1D wild-type strain, the NADPH-dependent glutamate dehydrogenase has more than two times higher specific activity relative to NAD^+-dependent glutamate dehydrogenase (GDH2) and NADH-dependent glutamate synthase (GLT1), which was coincided with the main responsibility of GDH1 for glutamate synthesis [23]. NADPH-dependent glutamate dehydrogenase activities of the *GDH1*-deleted strains were about four times lower than the activities of the wild-type and *GPD1*-disrupted strains. Residual activities of NADPH-dependent glutamate dehydrogenase were derived from an isozyme of GDH1 encoded by *GDH3* [19]. The disruption of NADH-consuming GPD1 led to a decrease in activities of both NADPH-consuming GDH1 and NADH-producing GDH2. It is probably ascribed to tight control of the NADH and glutamate levels inside the cells. No recycling of NADH by *GPD1* deletion drove NADH accumulation, allowing a decrease in NADH-producing GDH2 activity. This reduction seemed to change the level of glutamate, which influenced the expression of GDH1. Eventually, such a perturbation of NADH level decreased glycerol production. Then, by the deletion of the *GDH1* gene, NAD^+-dependent glutamate dehydrogenase (GDH2) activity decreased by 1.88-fold and 2.01-fold, and NADH-dependent glutamate synthase (GLT1) activity increased by 1.32 and 1.16-fold relative to the corresponding values for the wild-type and *GPD1*-disrupted strains, respectively. As observed in the activity assay of the wild-type *S. cerevisiae*, the glutamate level seems to be mainly controlled by the GDH1-catalyzing conversion of α-ketoglutarate. The disruption of NADPH-consuming GDH1 might trigger glutamate synthesis via glutamine reduction by NADH-consuming GLT1 (Fig. 1). Such NADH oxidation by the GLT1 reaction might substitute for the same NADH oxidation by GPD1, so the metabolic flux of dihydroxyacetone phosphate toward glycerol was directed to the ethanol production stream and hence an increase in ethanol yield was achieved in both the single deletion of *GDH1* and double deletion of *GPD1* and *GDH1*. However, a limited activity of GLT1 in *S. cerevisiae* led to insufficient oxidation of excess NADH caused by the *GPD1* and/or *GDH1* disruption, which might require more modification of GLT1 [23].

Conclusion

Glycerol and glutamate metabolisms were modified in *S. cerevisiae* in order to decrease glycerol production. Single deletion of *GPD1* or *GDH1*, and their double disruption

decreased glycerol production, especially the double deletion of *GPD1* and *GDH1* resulted in the lowest glycerol concentration of 2.31 g/L without affecting ethanol production. Perturbation of the intracellular pool of NAD(P)H by double deletion of *GPD1* and *GDH1* seemed to change the expression of other cofactor-related enzymes to recover the balance of the NAD(P)H pool and thereby to exert a synergetic effect on the reduction of glycerol production. More research is needed to elucidate the relationship between glycerol formation and ethanol production.

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