J Ind Microbiol Biotechnol (2011) 38:935–943
DOI 10.1007/s10295-010-0864-9

ORIGINAL PAPER

Improving ethanol productivity by modification of glycolytic redox factor generation in glycerol-3-phosphate dehydrogenase mutants of an industrial ethanol yeast

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Received: 26 July 2010 / Accepted: 25 August 2010 / Published online: 9 September 2010

Abstract The *GPD*2 gene, encoding NAD^+-dependent glycerol-3-phosphate dehydrogenase in an industrial ethanol-producing strain of *Saccharomyces cerevisiae*, was deleted. And then, either the non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) from *Bacillus cereus*, or the $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Kluyveromyces lactis*, was expressed in the obtained mutant AG2 deletion of *GPD*2, respectively. The resultant recombinant strain AG2A (*gpd*2Δ P_{PGK}-*gapN*) exhibited a 48.70 ± 0.34% (relative to the amount of substrate consumed) decrease in glycerol production and a 7.60 ± 0.12% (relative to the amount of substrate consumed) increase in ethanol yield, while recombinant AG2B (*gpd*2Δ P_{PGK}-*GAPDH*) exhibited a 52.90 ± 0.45% (relative to the amount of substrate consumed) decrease in glycerol production and a 7.34 ± 0.15% (relative to the amount of substrate consumed) increase in ethanol yield compared with the wild-type strain. More importantly, the maximum specific growth rates (μ_{max}) of the recombinant AG2A and AG2B were higher than that of the mutant *gpd*2Δ and were indistinguishable compared with the wild-type strain in anaerobic batch fermentations. The results indicated that the redox imbalance of the mutant could be partially solved by expressing the heterologous genes.

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Keywords *Saccharomyces cerevisiae* · Glycerol-3-phosphate dehydrogenase · $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase · Glycerol production · Ethanol yield

Introduction

As the reserves of petroleum decrease, ethanol, which is renewable and bio-based, has become one of the alternative liquid fuels to gasoline [24]. Glycerol is a main by-product consuming up to 5% of the carbon source in industrial ethanol fermentation [19]. To reduce the production of glycerol and lead carbon source flux towards the synthesis of ethanol is an important way to improve the ethanol yield. Formation of glycerol occurs in two steps from dihydroxyacetone phosphate (DHAP), catalyzed by glycerol-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphate phosphatase (GPP). The first step of glycerol formation, catalyzed by GPD, is rate-controlling [9, 21]. Two isogenes encoding different molecular forms of GPD have been identified. The first isoform of the GPD enzyme studied was found to be induced by osmotic stress [2] and was later demonstrated to be coded for by the *GPD*1 gene [18]. A second gene, *GPD*2, highly homologous to *GPD*1, was studied by Ansell et al. [3] and showed to be induced under anaerobic conditions. During anaerobic conditions, the respiratory chain does not function, ethanol production during fermentation is redox neutral, and glycerol formation by yeast is essential to reoxidize NADH, formed in the synthesis of biomass and secondary fermentation products, to NAD^+. Furthermore, glycerol is formed and accumulated inside the cells during growth under osmotic stress conditions, in which the compound functions as an efficient osmolyte protecting the cell against lysis [2, 3, 18]. Attempts to increase ethanol formation

during anaerobic growth by eliminating glycerol synthesis through deletions of *GPD*1 and *GPD*2, encoding two isoenzymes of glycerol-3-phosphate dehydrogenase in *S. cerevisiae*, were not successful, since the maximum specific growth rate was severely lowered in such strains [6, 14, 19, 23]. Introduction of new NAD^+ regeneration systems into yeast deletion of *GPD*1 and *GPD*2, for example, expression of nicotinamide nucleotide transhydrogenase, is a promising strategy until an enzyme possessing specific catalytic activity (for the following reaction: $NAD^+ + NADPH \rightarrow NADH + NADP^+$) is found.

Another possible metabolic engineering strategy for redirecting the flux of carbon from glycerol towards ethanol is to substitute the NAD^+-reducing reactions in biomass formation by $NADP^+$-reducing reactions to reduce the formation of NADH [8]. In the glycolytic pathway of the *S. cerevisiae*, one molecule of NADH is produced during 3-phosphoglycerate synthesis from the two coupled reactions, which are catalyzed by NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (NAD^+-GAPDH) and phosphoglycerate kinase (PGK) (Fig. 1). The most common form of GAPDH found in nature is the NAD^+ enzyme (EC 1.2.1.12). It was regarded as the only form present in mammalian tissue, yeast, and most bacteria for many years. Arnon et al. [4], however, found that the oxidation of glyceraldehyde-3-P in green leaves was catalyzed by more than one enzyme and they classified three forms of glyceraldehyde-3-P dehydrogenase as follows: (a) $NADP^+$-dependent enzyme (EC 1.2.1.9) that does not require phosphate and catalyzes an irreversible oxidation of glyceraldehyde-3-P to 3-phosphoglycerate; (b) NAD^+-dependent enzyme (EC 1.2.1.12) that requires phosphate and catalyzes a reversible reaction; and (c) $NADP^+$-dependent enzyme (EC 1.2.1.13) that also requires phosphate and catalyzes a reversible reaction. Thereafter, several bacterial $NADP^+$-specific glyceraldehyde-3-P dehydrogenases were found and characterized [10, 16, 17]. Recently, the first eukaryotic, nonplant, $NADP^+$-linked GAPDH from *Kluyveromyces lactis* was identified by Verho et al. [28]. This was the first and only $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase found in yeast until now.

Overexpression of non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) could decrease the formation of NADH during the glycolysis by substituting the $NADP^+$-reducing reactions for NAD^+-reducing reactions and thereby reduce the production of glycerol and improve the ethanol yield [8]. In the present study, *GPD*2 was deleted to reduce the glycerol production at first. And then, in order to relieve the toxic effects of surplus NADH on yeast cells, GAPN from *Bacillus cereus* was expressed in the obtained mutant *gpd*2Δ to overcome the problem of the redox imbalance.

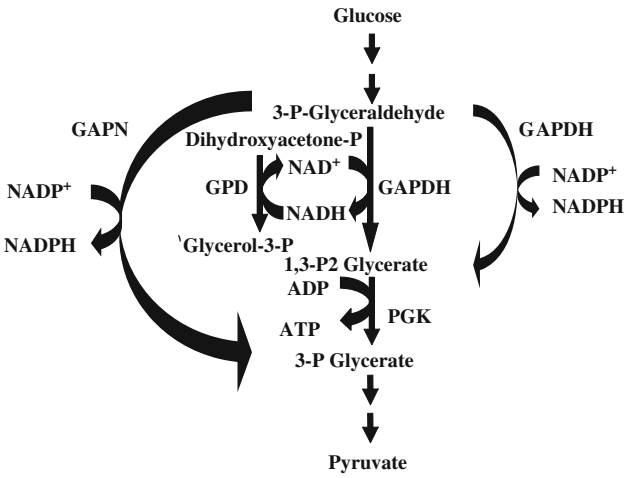

Fig. 1 Biochemical reaction catalyzed by GAPN, $NADP^+$-GAPDH, and GPD shown together with the endogeneous reactions of the Embden–Meyerhof–Parnas pathway

To investigate its expression efficiency in yeast, a eukaryotic origin $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GAPDH) from *K. lactis* was also expressed. To our knowledge, this is the first study about the application of overexpression of GAPN and $NADP^+$-GAPDH in *GPD*2-deleted industrial ethanol-producing yeast.

Materials and methods

Yeast strains and media

The genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. *S. cerevisiae* haploid strains ANGA1 (*MATa*) and ANGA2 (*MATα*) used in this study for genetic manipulation were derived from the polyploid *S. cerevisiae* CICIMY0086 (an industrial ethanol-producing yeast; http://cicim-cu.sytu.edu.cn/). Strains were routinely grown in a medium composed of 1% yeast extract, 2% Bacto peptone, and 2% glucose (YPD); solid media contained 2% agar. Incubation conditions were standardized on the rotary shaker at 30°C with 150 rpm.

Construction of the plasmids

Deletion of GPD2

All primers used in this study are listed in Table 2. The gene *GPD*2, encoding glycerol-3-phosphate dehydrogenase in *S. cerevisiae*, was cloned by PCR amplification from industrial *S. cerevisiae* CICIMY0086 using primers GPD2F and GPD2R. A 2,107-bp PCR fragment including the *GPD*2 promoter and terminator was obtained and

Table 1 Microbial strains and plasmids used in the present study

	Relevant genotype	Source of reference
Strains		
S. cerevisiae	Polyploid, wild type	CICIMY0086, JU
S. cerevisiae ANGA1	*MATa*, wild type	Guo et al. [14]
S. cerevisiae ANGA2	*MATα*, wild type	Guo et al. [14]
S. cerevisiae GA1	*MATa*, *gpd2*::*kan*^{r}	This investigation
S. cerevisiae GA2	*MATα*, *gpd2*::*kan*^{r}	This investigation
S. cerevisiae AG2	Polyploid, *gpd2*Δ::*kan*^{r}	This investigation
S. cerevisiae AG2A	Polyploid, *gpd2*Δ P_{PGK}-*gapN*	This investigation
S. cerevisiae AG2B	Polyploid, *gpd2*Δ P_{PGK}-*GAPDH*	This investigation
B. cereus	Wild type	CICIM B0627, JU
K. lactis	Wild type	CICC1773
E. coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* (*Lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*M15	Strategene
Plasmids		
pMD18-T simple	*bla*[a]	TaKaRa, Japan
pMG2	*bla GPD2*	This investigation
pMG2-*kan*^{r}	*bla* $GPD2_P$-*loxp-kan-loxp*-$GPD2_t$	This investigation
pMGKR	*bla kan* $PGK1_P$-$PGK1_t$-*rDNA*	This investigation
pMGKR-*gapN*	*bla kan* $PGK1_P$-*gapN*-$PGK1_t$-*rDNA*	This investigation
pMGKR-*gapdh*	*bla kan* $PGK1_P$-*gapdh*-$PGK1_t$-*rDNA*	This investigation

[a] β-Lactamase gene, confers resistance to penicillin in *Escherichia coli* JM109

Table 2 Sequences of the oligonucleotide primers used in this study

Primer names	Sequence 5′-3′ (restriction sites are underlined)	Restriction sites
GPD2F	TAA*GGATCC*[a]ATGTAATAAGCAAACAAGCACGAATG	*Bam*HI[a]
GPD2R	TTA*GGATCC*[a]TCGTCAACTTCT CTGCATGTGATTAT	*Bam*HI[a]
GPD2V1	ATAATTACCG*CCCGGG*[a]TGA TAAGGAAGGGGAGCG	*Sma*I[a]
GPD2V2	ATA*CCCGGG*[a]AGGCCTAGACCT TACTTCCACGTCAA	*Sma*I[a]
PF1	ATTTTAGATTCCTGACTTCAACTC	
PR2	GCG*GTCGAC*[a]*GGATCC*[b]TGTTTTATATTTGTTGTAAAAAG TAG	*Sal*I[a], *Bam*HI[b]
TF1	CCG*GTCGAC*[a]TTCTTTGGAATTATTGGAAGGTA	*Sal*I[a]
TR2	GCG*GCGGCCGC*[a]GAACGCAGAATTTTCGAGTTAT	*Not*I[a]
KF1	AG*GCGGCCGC*[a]*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b]GCCCAGTAGTAGGTTGAGG	*Not*I[a], *loxp*[b]
KR2	AG*GCGGCCGC*[a]*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b]TTGAAGTCGGACAGTGAGT	*Not*I[a], *loxp*[b]
GBF1	ACAT*GGATCC*[a]ATGACAGAACACTATTTAAACTATG	*Bam*HI[a]
GBR2	GTGA*GTCGAC*[a]TTAGTCTTCGATGTTGAAGACA	*Sal*I[a]
GKF1	GGC*GGATCC*[a]ATGCCCGATATGACAAACG	*Bam*HI[a]
GKR2	CCG*GTCGAC*[a]TTAAACACCAGCTTCGAAGTC	*Sal*I[a]
RF1	CCG*CATATG*[a]CTCTATCCCCAGCACGA	*Nde*I[a]
RR2	CCC*CATATG*[a]GAGAAACGGCTACCACATC	*Nde*I[a]

[a,b] Restriction site with corresponding restriction enzyme

inserted into pMD18-T simple vector resulting in plasmid pMG2. And then, the fragment containing *GPD2* promoter and terminator was amplified from the plasmid pMG2 by inverse PCR using the primers GPD2V1 and GPD2V2. The fragment was digested by *Sma*I and ligated with kanamycin resistance gene, which confers resistance to Geneticin in *S. cerevisiae* isolated from the vector pPIC9K with primers KF1 and KR2 containing 34-bp *loxp* site on both 5′-ends. The resultant plasmid was designated as pMG2-*kan*^{r}.

After that, the haploid strains ANGA1 (*MATa*) and ANGA2 (*MATα*) were transformed with the *Bam*HI-

digested fragment *gpd2*::*kan*^{r} from the plasmid pMG2-*kan*^{r}. The desired orientation of each insert was verified by PCR, resulting in strains GA1 (*MATa*, *gpd2*::*kan*^{r}) and GA2 (*MAT*, *gpd2*::*kan*^{r}). Polyploid mutant AG2 (*gpd2*Δ::*kan*^{r}) was constructed by crossing the above two haploid mutants followed with sporulation experiment. Finally, the *cre*-recombinase expression vector pSH47 [13] was transformed to the above mutant AG2 and the excision of the *kan*^{r} gene was verified by PCR.

Expression of GAPN and NADP$^+$-GAPDH

The plasmids used for yeast expression of GAPN and NADP$^+$-GAPDH were constructed as follows: *PGK1* promoter and terminator were cloned by PCR amplification from *S. cerevisiae* with primers PF1 and PR2, TF1 and TF2. After being digested by *Sal*I, the above two fragments were inserted into the pMD18-T simple vector to create plasmid pMG. Kanamycin resistance gene obtained previously was inserted into the *Not*I site of pMG. As there are approximately 200 rDNA-repeats in *S. cerevisiae*, part of the 18S ribosomal RNA coding sequence (an rDNA fragment) in these repeating regions was cloned and used as homologous integration sites to increase copy numbers with primers RF1 and RF2, and inserted into the *Nde*I site of pMG1, resulting in plasmid pMGKR. The two genes, encoding non-phosphorylating NADP$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) and NADP$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP$^+$-GAPDH), were obtained from *B. cereus* and *K. lactis* genomic DNA by PCR amplification using primers GBF1 and GBF2, GKF1 and GKF2, respectively. After being digested by *Bam*HI/*Sal*I, the two fragments were inserted into the plasmid pMGKR, resulting in plasmid pMGKR-*gapN* and pMGKR-*gapdh*, respectively.

Finally, the plasmids pMGKR-*gapN* and pMGKR-*gapdh* were cleaved with *Sac*II and, after being purified, the resultant linear DNA fragment was introduced into the mutant AG2 by the lithium acetate method [15]. For initial selection of yeast transformants, G418 was added with the final concentration of 0.25 mg/ml. Transformants with the G418$^+$ phenotype were isolated. Subsequently, a higher concentration of G418 (nearly 0.5 mg/ml), which provides a stronger selective pressure and may lead to higher copy numbers, was used [29]. Correct insertion of the gene encoding GAPN and NADP$^+$-GAPDH into the rDNA locus was verified by PCR.

Enzyme assay

The recombinants were cultured for 72 h at 30°C and the culture supernatant was isolated by centrifugation at 8,000*g* for 5 min to determinate the in vitro enzyme activity. The cells were collected and washed with buffer (50 mM lactic acid/sodium lactate buffer, pH 3.0) and disrupted by using a sonic dismembrator (VC750, Sonics, USA) with 30% of the total working energy for 5 min at 0°C to determine the enzyme activity. Activity of NADP$^+$-dependent GAPN was measured in a reaction mixture containing 125 mM triethanolamine (pH 8.3), 5 mM 2-mercaptoethanol, 1 mM NADP$^+$, and 0.1 ml of diluted enzyme sample, and the reaction was started by adding DL-glyceraldehyde-3-phosphate to a concentration of 0.5 mM [10]. Activity of NADP$^+$-dependent GAPDH was measured in a reaction mixture containing 125 mM triethanolamine (pH 8.3), 5 mM cysteine/HCl, 5 mM KH_2PO_4 (pH 7.5), 1 mM NAD$^+$, and 0.1 ml of diluted enzyme sample. The reaction was started by adding DL-glyceraldehyde-3-phosphate to a concentration of 0.5 mM as also described by Crow and Wittenberger [10]. Enzyme activities were assayed at 30°C by following the initial linear NADPH production, respectively, in a 1-ml reaction mixture at 340 nm using a spectrophotometer (CS-9301 PC densitometer, Tokyo, Japan). Measurements of enzyme activities were carried out in triplicate.

Cultivation conditions

Cultivations were carried out under oxygen limited conditions in a 1-L shake flask containing 500 ml medium. In order to maintain anaerobic conditions in batch fermentations, the shake flasks were plugged with a rubber stopper fitted with a vent pipe connected to a sterile water trap. Yeast cells were pre-cultured in 500-ml Erlenmeyer flasks at 30°C in YPD medium until OD_{600nm} achieved 10.0. This exponential growth phase culture was used to inoculate the fermentation medium to yield an initial OD_{600nm} of 0.4 (0.30 mg/ml dry mass). The medium for fermentation contains 50 g/l glucose and supplemented with 7.5 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, and trace metals and vitamins as described previously [26]. About 300 μl antifoam per liter was added to avoid foaming, and the medium was supplemented with 420 mg Tween 80 and 10 mg ergosterol per liter, which are necessary for anaerobic growth of *S. cerevisiae* [25]. During the fermentation process, the flasks were stirred with a magnetic stirrer and kept at 30°C in a thermostatic chamber. Fermentation experiments were performed in triplicate.

Analysis of product formation and determination of dry weight

The concentrations of glucose, ethanol, glycerol, pyruvic acid, and acetic acid in filtered samples withdrawn from the batch cultivations were determined by high-performance liquid chromatography (HPLC) with a SH1011 column

(Agilent, USA), at a column temperature of 50°C with 0.01 M H_2SO_4 as mobile phase at a flow rate of 0.8 ml/min and subsequently detected with a refractive index detector (Agilent, USA). The biomass concentration in the medium was measured gravimetrically as described earlier [20]. Two samples (10 ml each) were centrifuged at 6,000*g* for 15 min and washed twice with water and subsequently dried at 100°C for 24 h and weighed. The product yield was calculated as the ratio of the amount of product obtained divided by the amount of substrate consumed. Glucose consumption rate was defined as the amount of glucose consumed per hour during the fermentation process.

Protein determination

Two samples (10 ml each) were centrifuged at 6,000*g* for 5 min and washed twice with 0.9% (w/v) NaCl. The pellets were resuspended in 3 ml NaOH and total protein was determined by a modified biuret method using bovine serum albumin as a standard [22, 27].

Results

Overexpression of GAPN and $NADP^+$-GAPDH in *GPD2*-deleted mutant

The genomic DNA analysis showed that the disruption cassette *gpd2*::*kan*^{r} was correctly incorporated into the *GPD2* locus of the chromosome of *S. cerevisiae* and the resultant mutant was named AG2. And the cassette for expressing GAPN or GAPDH, successfully introduced into the above mutant deletion of glycerol-3-phosphate dehydrogenase encoding by *GPD2*, was also verified by PCR. Subsequently, the $NADP^+$-dependent GAPN and $NADP^+$-dependent GAPDH activities of 20 transformant colonies were investigated. Two recombinants denoted AG2A (*gpd2*Δ P_{PGK}-*gapN*) and AG2B (*gpd2*Δ P_{PGK}-*GAPDH*) showed apparently high activities (4.15 U/mg protein for AG2A and 3.05 U/mg protein for AG2B) compared to the wild-type strain, which produced no detectable activity, and were used in the subsequent experiments. In order to overexpress the heterologous gene (*gapN* and $NADP^+$-*GAPDH* in this study), a higher concentration of G418, nearly 0.5 mg/ml, was used as a selective pressure. Our previous study had shown that a single copy of the gene integrated into the genome conferred resistance to Geneticin to a level of ca. 0.25 mg/ml. Multiple integrated copies of the gene can increase the Geneticin resistance level from 0.5 mg/ml (1–2 copies) up to 2 mg/ml (4–8 copies). Analyzing the enzyme activity was the first step to select the transformants with higher copy numbers of the genes and Southern blot analysis showed that the two recombinants AG2A and AG2B contained two copies of the genes, respectively, integrated into their genome (data not shown).

Growth characteristics

The effects on the cellular physiology of the introduced genetic changes of *S. cerevisiae* CICIMY0086 (wild type), resulting in strains AG2 (*gpd2*Δ), AG2A (*gpd2*Δ P_{PGK}-*gapN*), and AG2B (*gpd2*Δ P_{PGK}-*GAPDH*), as well as the wild type used as the control, were studied under anaerobic growth conditions. Several parameters including growth rate, biomass, and protein content were analyzed. As shown in Table 3, the results showed that deletion of *GPD2* apparently resulted in a decline in the maximum specific growth rate (μ_{max}) for the strain AG2 (0.19 h^{-1}) which was consistent with earlier studies (Fig. 2a) [6, 23]. However, when the heterologous gene *gapN* or $NADP^+$-*GAPDH* was introduced into the mutant, μ_{max} could rise to its original level nearly the same as the wild type. On the other hand, the biomass yields of AG2A and AG2B were slightly lower than the type strain; whereas for the mutant, a dramatic decrease in biomass yield (nearly 20.7% lower than the parent strain) was observed. Besides, less cellular protein content of mutant AG2 was detected at the end of fermentation; such a decrease might have resulted from the imbalance of redox state of the yeast when *GPD2* was deleted (Table 3), since synthesis of protein was a major cellular activity to produce surplus NADH and the mutant in some way managed to maintain redox balance. Theoretically, an effective way of solving the redox problem in yeast is to ensure that the biomass contains less protein [6].

Comparison of substrate consumption and production formation

Deletion of *GPD2* resulted in lower substrate consumption rate for the mutant AG2 (1.19 ± 0.16 g/h). Meanwhile, the specific glucose uptake rate of all three recombinant strains with lower cell density was higher than wild type, indicating that their biomass synthesis ability was hampered by the introduced mutation (Table 3). Overexpression of GAPN or $NADP^+$-GAPDH in mutant AG2 had a significant effect on glucose consumption rate which increased to 1.67 ± 0.13 g/h and 1.72 ± 0.21 g/h for AG2A and AG2B, respectively, close to the wild type (1.72 ± 0.18 g/h) and much higher than that of the mutant AG2 (Fig. 2b). Meanwhile, the lower glucose consumption rate of AG2 made the fermentation period prolong for 10 h at least, apparently a disadvantage for ethanol production. The lower substrate consumption rate for the mutant deletion of *GPD2* may result from the poor growth ability of the strain as shown in Fig. 2a. Besides, the type strain produced more

Table 3 Comparison of growth and compound yields of *S. cerevisiae* CICIMY0086 wild type, AG2 (*gpd2*Δ), AG2A (*gpd2*Δ P_{PGK}-*gapN*), and AG2B (*gpd2*Δ P_{PGK}-*GAPDH*) respectively, during anaerobic batch growth on 5% glucose

Parameter	AG2[a]	AG2A[a]	AG2B[a]	Wild type[a]
μ_{max} (h^{-1})	0.19 ± 0.02	0.27 ± 0.01	0.28 ± 0.01	0.29 ± 0.02
v_{glc} (g/g/h)	0.517 ± 0.001	0.459 ± 0.002	0.488 ± 0.001	0.446 ± 0.002
$Y_{X/S}$ (g/g)	0.058 ± 0.001	0.068 ± 0.002	0.064 ± 0.001	0.070 ± 0.002
$Y_{P/X}$ (g/100 g)	46.32 ± 1.4	50.75 ± 1.0	49.87 ± 1.6	51.22 ± 1.1
$Y_{Eth/S}$ (g/g)	0.50 ± 0.01	0.502 ± 0.01	0.501 ± 0.02	0.467 ± 0.02
$Y_{Ace/S}$ (mg/g)	2.37 ± 0.14	3.48 ± 0.26	3.56 ± 0.17	3.59 ± 0.24
$Y_{Gly/S}$ (g/g)	0.050 ± 0.002	0.037 ± 0.002	0.034 ± 0.001	0.073. ± 0.003
$Y_{Pyr/S}$ (mg/g)	0.357 ± 0.04	0.579 ± 0.03	0.583 ± 0.02	0.610 ± 0.04
Residual sugar (g/l)	1.99 ± 0.23	ND	ND	ND

Data are presented as ± the standard deviation

ND not detectable; μ_{max}, maximum growth rate; v_{glc}, specific glucose uptake rate (g glucose/g dry cell weight/h); $Y_{X/S}$, biomass yield (g DW/g glucose); $Y_{P/S}$, protein (g/100 g biomass); $Y_{Eth/S}$, ethanol yield (g ethanol/g glucose); $Y_{Ace/S}$, acetate yield (mg acetate/g glucose); $Y_{Gly/S}$, glycerol yield (g glycerol/g glucose); $Y_{Pyr/S}$, pyruvic acid (mg pyruvic acid/g glucose)

[a] Triplicate experiments

glycerol during the whole fermentation process than the other strains. Fast ethanol production occurred in the initial 16 h, in accordance with the exponential growth phase of all the strains. Although the average ethanol formation rate of the mutant AG2 could reach 0.730 ± 0.003 g/h, faster than that of the wild type (0.751 ± 0.003 g/h), its ethanol production rate in the initial 12 h was lower (Fig. 2d). The ethanol formation rate for AG2A and AG2B was 0.786 ± 0.002 and 0.784 ± 0.002, respectively. The above results clearly demonstrated that the poor performance of the mutant caused by deletion of *GPD*2 resulting in redox imbalance can be partly restored by expressing a high specific activity of GAPN or $NADP^+$-GAPDH.

Analysis of fermentation product yields

HPLC analysis of the fermentation products revealed that by deletion of *GPD*2, the ethanol yield for strain AG2 (*gpd2*Δ) increased by 7.19 ± 0.21% (relative to the amount of substrate consumed) while glycerol formation reduced by 30.80 ± 0.52% (relative to the amount of substrate consumed) compared to the wild type during anaerobic batch fermentations (Table 3). As reported in a previous study [8], overexpression of GAPN in a laboratory strain of *S. cerevisiae* resulted in a 40% lower glycerol yield on glucose while the ethanol yield increased with 3% without affecting the maximum specific growth rate. Similarly, in the case of the industrial yeast we used in this study, either expression of GAPN or $NADP^+$-GAPDH could decrease the glycerol yield by about 30%, and improve the ethanol yield by 3.0% (data not shown); however, such an increase was highly dependent on the expression level and was unstable, especially when an industrial wild-type strain was used. Expression of *gapN* or $NADP^+$-*GAPDH* in *GPD*2-deleted mutant AG2 resulted in a 7.60 ± 0.12% and 7.34 ± 0.15% increase in ethanol yield, respectively. Although such an increase in ethanol yield was not so apparent compared with that of mutant AG2, significant decreases in glycerol yield were observed for AG2A and AG2B (48.70 ± 0.34% and 52.90 ± 0.45%, respectively) (Table 3). Meanwhile, detection of main by-products illustrated that the concentrations of acetate and pyruvic acid were basically indistinguishable for the mutant AG2A and AG2B compared with the wild type. However, the mutant AG2 exhibited a 24.8 and 41.5% decrease in yields of acetate and pyruvic acid, respectively (Fig. 3). Thus changes in the synthesis of by-products might be another example of metabolic adjustment by the cells to minimize the NADH surplus when the glycerol production capacity was hampered.

Discussion

Under aerobic conditions, the surplus NADH formed in metabolic reactions is reoxidized to NAD^+ by mitochondrial respiration [5]. In contrast, under anaerobic conditions, glycerol formation by yeast is essential to reoxidize NADH, which is formed in the synthesis of biomass and secondary fermentation products, to NAD^+ [1, 11]. One way to mediate the redox balance in yeast deletion of *GPD*1 or *GPD*2 is by regulating redox balance in ammonia metabolism by over-expressing the *GLT*1 and *GLN*1 genes, encoding glutamate synthase and glutamine synthase, respectively, and deletion of gene *GDH*1, which encodes the NADPH-dependent glutamate dehydrogenase, but the growth of the strain was

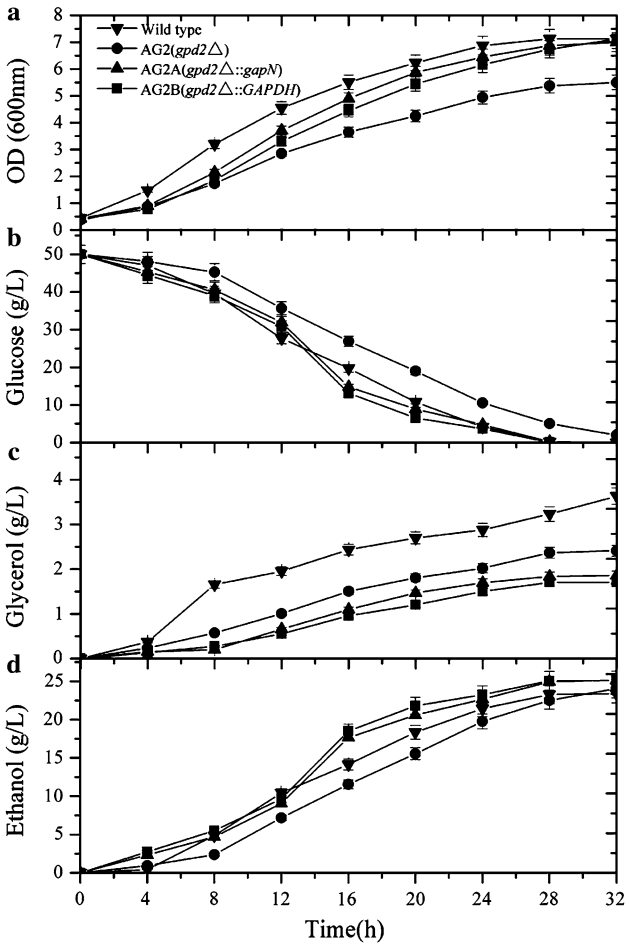

Fig. 2 Changes in measured parameters during anaerobic batch cultures of *S. cerevisiae* CICIMY0086 wild type, AG2 (*gpd*2Δ), AG2A (*gpd*2Δ P_{PGK}-*gapN*), and AG2B (*gpd*2Δ P_{PGK}-*GAPDH*) respectively, with 5% glucose as carbon and energy source. Shown are **a** OD_{600nm}, optical density at 600 nm, **b** glucose, **c** glycerol, and **d** ethanol concentrations versus time

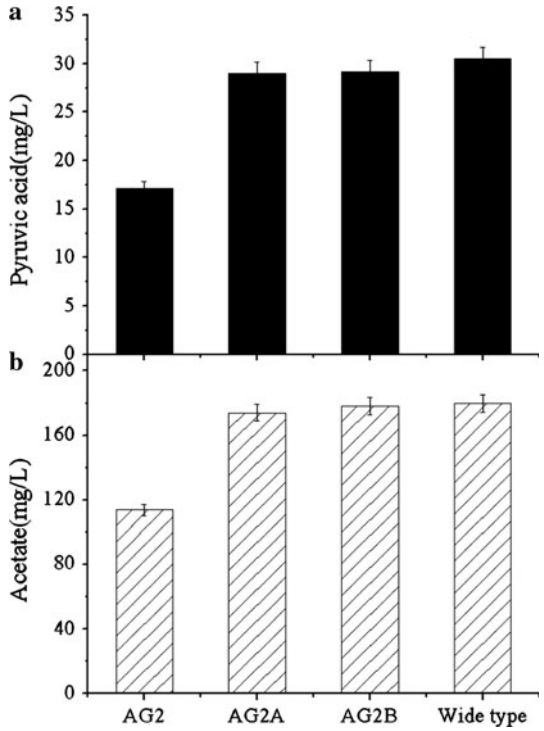

Fig. 3 Changes in measured parameters during anaerobic batch fermentation with 5% glucose as carbon and energy source. **a** Concentration of pyruvic acid in the medium of *S. cerevisiae* CICIMY0086 wild type, AG2 (*gpd*2Δ), AG2A (*gpd*2Δ P_{PGK}-*gapN*), and AG2B (*gpd*2Δ P_{PGK}-*GAPDH*) respectively. **b** Concentration of acetate in the medium of wild type, AG2 (*gpd*2Δ), AG2A (*gpd*2Δ P_{PGK}-*gapN*), and AG2B (*gpd*2Δ P_{PGK}-*GAPDH*) respectively

severely affected [19]. A recent study by Guadalupe Medina et al. [12] showed that glycerol production could be completely eliminated by engineering *S. cerevisiae* (*gpd*1Δ *gpd*2Δ) such that it can reoxidize NADH by the reduction of acetic acid to ethanol via NADH-dependent reactions. However, growth and product formation in the engineered strain were significantly slower than the parent strain. Therefore, completely eliminating the production of glycerol is not practical until now, since it is the precursor used for synthesizing the cellular membrane and plays an important role in maintaining cellular redox balance. Besides, it is the main osmoregulator in *S. cerevisiae* under hyperosmotic conditions [7]. And we believe the best strategy to improve the ethanol yield is to knockdown, not knockout, the glycerol pathway.

In the present study, the effects of expression of non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN), or $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mutant AG2 (*gpd*2Δ) were investigated. Deletion of *GPD*2 in *S. cerevisiae* gave a nearly 30% reduction in glycerol formation and a 7% increase in ethanol compared to the wild type. How the redox balance in mutant AG2 (*gpd*2Δ) is accomplished is not known; part of the explanation is a decrease in the amount of by-product formed. There must, however, be additional mechanisms as well. Meanwhile, consistent with early studies [4, 6, 23], lower growth rate, decline in the protein content of the cells, and dramatic reduction in pyruvic acid and acetate production were observed in the *gpd*2Δ mutant, and these changes might reflect the cells ability to recognize the reduction in NADH-oxidizing capacity when the gene was deleted. Overexpression of heterologous GAPN or $NADP^+$-GAPDH could decrease the formation of NADH in glycolysis by substituting the $NADP^+$-reducing reactions for NAD^+-reducing reactions catalyzed by yeast NAD^+-GAPDH, thereby reducing the production of glycerol. The comparatively lower improvement on ethanol yield when the genes were expressed in recombinant strains AG2A and AG2B indicated that its main effects were to restore the growth of the *GPD*2-deleted

mutant. This inference was confirmed by the redirection of the carbon source flux towards biomass synthesis and organic acid as can be seen from higher biomass yield and pyruvic acid and acetate yields of two recombinants. Therefore, μ_{max} could achieve its original level. In addition, the reduction in glycerol yield of the mutant AG2A was lower than that of AG2B, while its increase in ethanol yield was higher than AG2B. The reason for this phenomenon might due to the different reaction catalyzed by the two enzymes. In wild-type *S. cerevisiae*, the conversion of glyceraldehyde-3-phosphate into 3-phosphoglycerate is a two-step reaction catalyzed by NAD^+-dependent GAPDH (EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3) converting NAD^+ into NADH and ADP to ATP, respectively. GAPN (EC 1.2.1.9) catalyzes the irreversible oxidation of glyceraldehyde-3-phosphate and $NADP^+$ into 3-phosphoglycerate and NADPH in glycolysis (Fig. 1), and no amount of ATP being released compared with $NADP^+$-GAPDH, which could result in higher yield of biomass and ethanol [8].

Furthermore, though many efforts have been made to interrupt the glycerol pathway to improve the ethanol production, the strains they used were either auxotrophic or laboratory strains. In order to move towards practical ethanol production, the development of genetically engineered industrial strains should be promoted more strongly. The industrial ethanol-producing *S. cerevisiae* CICIMY0086 is polyploid and usually contains more than two copies of one single gene in its genome. It is too difficult to delete all the copies for one gene in this strain. Thus, the haploid strain isolated from the industrial strain in our previous study was used for genetic manipulation [14]. After that, polyploid genetic type was recovered by crossing the haploid strains of different mate type. Otherwise, it is difficult to introduce several heterologous genes into the wild-type strain due to the limited marker genes available for selection. In this study, a gene deletion vector was constructed and the *loxP* sites were added to the ends of the selecting marker according to a strategy described by Güldener et al. [13]. The marker can be excised by *cre*-recombinase making it possible to conduct further genetic changes by reusing this selecting marker.

Conclusion

Our results have shown that by overexpression of GAPN or $NADP^+$-GAPDH in mutant deletion of *GPD*2, the toxic effect of surplus NADH could be relieved by reducing its production in glycolysis, further reducing the production of glycerol and improving the ethanol yield without affecting the growth and fermentation ability of the yeast. Therefore, the combination of the expression of GAPN or $NADP^+$-GAPDH with the deletion of *GPD*2 was an effective approach to increase the ethanol yield.

Acknowledgments This work was financial supported by the Natural Science Foundation of China (20706024), the "863" Program (2006AA020101, 2007AA10Z359), Innovative Research Team of Jiangsu Province and China Postdoctoral Science Foundation funded project (200801361).

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