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Overexpressing *GLT1* in *gpd1*Δ mutant to improve the production of ethanol of *Saccharomyces cerevisiae*

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Abstract To improve ethanol production in *Saccharomyces cerevisiae*, two yeast strains were constructed. In the mutant, KAM-4, the *GPD1* gene, which encodes a glycerol 3-phosphate dehydrogenase of *S. cerevisiae* to synthesize glycerol, was deleted. The mutant KAM-12 had the *GLT1* gene (encodes glutamate synthase) placed under the *PGK1* promoter while harboring the *GPD1* deletion. Notably, overexpression of *GLT1* by the *PGK1* promoter along with *GPD1* deletion resulted in a 10.8% higher ethanol production and a 25.0% lower glycerol formation compared to the wild type in anaerobic fermentations. The growth rate of KAM-4 was slightly lower than that of the wild type under the exponential phase whereas KAM-12 and the wild type were indistinguishable in the biomass concentration at the end of growth period. Meanwhile, dramatic reduction of formation of acetate and pyruvic acid was observed in all the mutants compared to the wild type.

Keywords Ethanol production · Glutamate-synthase gene · Glycerol formation · Glycerol 3-phosphate dehydrogenase · *S. cerevisiae*

Introduction

During the production of ethanol by *S. cerevisiae*, glycerol, which is one of the main byproducts, is produced at up to 5 g/l and accounts for up to 5% of the carbon source in industrial fermentations (Nissen et al. 2000). Glycerol is synthesized from the glycolytic intermediate dihydroxyacetone phosphate in two steps catalyzed by nicotinamide adenine dinucleotide (NAD^+)-dependent glycerol 3-phosphate dehydrogenase (Gpdp), followed by dephosphorylation of glycerol 3-phosphate catalyzed by glycerol 3-phosphatase (Gppp) (Norbecket al. 1996; Pahlman et al. 2001; Remize et al. 2001). Glycerol plays a biological role in maintaining cytosolic redox balance (Bakker et al. 2001). Ethanol production is redox-neutral, but the surplus cytosolic NADH produced during other cellular processes is reoxidized by Gpdp to avoid a serious imbalance in the NAD^+/NADH ratio. Glycerol is also involved in the osmoregulation of *S. cerevisiae* as the main compatible solute accumulated in the yeast cells (Hohmann 2002).

Ammonium is assimilated into glutamate by reaction with α-oxoglutarate catalyzed by an NADPH-dependent glutamate dehydrogenase encoded by *GDH1*. Another system that synthesizes glutamate exists in *S. cerevisiae*. This consists of two coupled reactions, catalyzed by glutamate synthase, encoded by *GLT1*, and glutamine synthetase, encoded by *GLN1* (Torben et al. 2000). It should be possible to convert NADH to NAD^+ in the synthesis of glutamate from ammonium and α-oxoglutarate resulting in a reduced surplus formation of NADH by overexpressing *GLT1*, resulting to lower glycerol production (Torben et al. 2000).

Despite the number of strategies adopted for the construction of the ethanol-producing yield of yeast strains of *S. cerevisiae*, there have been no reports about the application of overexpressing *GLT1* in *gpd1*Δ of *S. cerecisiae* (Michnick et al. 1997; Remize et al. 1999; Oner et al. 2005). Hence, the purpose of this investigation is to decrease glycerol formation and improve ethanol

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production in *S. cerevisiae* through knocking-out of *GPD1* and overexpressing *GLT1* to consume NADH under anaerobic fermentation conditions.

Materials and methods

Yeast strains and growth conditions

S. cerevisiae haploid strains used in this study were derived from the diploid industrial strains TH-AADY (Angel Yeast, China). Strains KAM-4 (*MATα ura3 gpd1*Δ::REPEAT) and KAM-12 (*MATα ura3 gpd1*Δ::REPEAT P_{PGK}-*GLT1*) were constructed in this study. Yeast cells were grown in medium (YEPD). YNB without amino acids (Difco) supplemented with 2% (*w*/*v*) glucose and amino acids according to the strains demands and 5-fluoroorotic acid medium (5-FOA) were used for yeast growth and selection of transformants (Sambrook et al. 1989). Incubation conditions were standardized on the rotary shaker at 30°C with 200 rpm.

One-step disruption of GPD1

Cassette GRYURG was constructed for disrupting *GPD1*, which contains, from left to right, part of 5′-end of the *GPD1* coding sequence, a short fragment (denoted R) of *B. subtilis* chromosomal DNA, the *URA3* gene, an inverted R fragment, and a segment of the 3′-end region of the *GPD1*. The plasmid pUC18-R was created by inserting a 446-bp of chromosomal DNA from *B. subtilis* 168 to pUC18. The 446-bp fragment was amplified by polymerase chain reaction (PCR) using primers R0011F and R0012R that contain *Bam*HI and *Xba*I sites, respectively. All primers used in this study are listed in Table 1. Digestion of both the PCR product and pUC18 by *Bam*HI and *Xba*I and subsequent ligation resulted in plasmid pUC18-R. Then, the *URA3* marker was amplified from YEPlac195 by primers U0011F and U0012R. After digestion with both *Xba*I and *Sal*I, the *URA3* gene was cloned to the same sites of pUC18-R, yielding plasmid pUC18-RYU. Subsequently, the R fragment was amplified again from *B. subtilis* 168 with primers R0013F and R0014R, and the *Sal*I and *Pst*I portion was inserted next to the right of *URA3* in pUC18-RYU to generate plasmid pUC18-RYUR. Finally, two primers, KGPD1U and KGPD1D, were used to amplify the RYUR disruption cassette from plasmid pUC18-RYUR. Primer KGPD1U contains 20 nucleotides derived from the plasmid pUC18-RYUR at the 3′ end, which is flanked by 40 nucleotides (601 to 640 bp) downstream of the ATG start codon of *GPD1* at the 5′ end. Similarly, KGPD1D contains 20 nucleotides of complementary strand of the plasmid pUC18-RYUR at the 3′ end, flanked by 40 nucleotides (1,216 to 1,177 bp) of the complementary strand downstream of the ATG start codon of *GPD1* at the 5′ end. The resulting PCR products of 1,360 bp in length were used to transform into the wild type strain KAM-2 (*Mat αura3*) to disrupt *GPD1*. After growing on CM minus uracil for 2–3 days, transformants containing correct deletion of *GPD1* were verified by PCR with primers CGPD1U and CGPD1D. Primer CGPD1U contains nucleotides 466 to 447 upstream

Table 1 List of primers used in this study

Primer names	Primer sequences	Restriction sites
R0011F	5′-GGG CCC GGA TCC GAG CAG CAT AAA CGA CTG CT-3′	*BamHI*
R0012R	5′-GGG CCC TCT AGA ACG CTC AAT GTT GTT CAT GA-3′	*XbaI*
U0011F	5′-GGG CCC TCT AGA GTA GTC TAG TAC CTC CTG TG-3′	*Xba*I
U0012R	5′-GGG CCC GTC GAC GAA AAG TGC CAC CTG ACG TC-3′	*Sal*I
R0013F	5′-GGG CCC GTC GAC GAG CAG CAT AAA CGA CTG CT-3′	*Sal*I
R0014R	5′-GGG CCC CTG CAG ACG CTC AAT GTT GTT CAT GA-3′	*Pst*I
KGPD1U	5′CAC ATT CCA AAG GAT TTC AGA GGC GAG GGC AAG GAC GTC GAC GAC GTT GTA AAA CGA CG3′	
KGPD1D	5′AGT GGG GGA AAG TAT GAT ATG TTA TCT TTC TCC AAT AAA TGG AAA CAG CTA TGA CCA TG3′	
CGPD1U	5′CAATGAGACTGTTGTCCTCC3′	
CGPD1D	5′GCATGCAAGCTTGAGTGGTGTTGTAACCACCC3′	
GT001F	5′-GGG CCC GTC GAC ATG CCA GTG TTG AAA TCA GA-3′	*Sal*I
GT002R	5′-GGG CCC CTG CAG TTT TAG TAT CGA CCA TTT CA-3′	*Pst*I
GTP01F	5′-GGG CCC GGT ACC TTT CTG AGC ACT GTC AGG AG-3′	*Kpn*I
GTP02R	5′-GGG CCC GGA TCC TGA TTT CA A CAC TGG CAT GC-3′	*Bam*HI
PK001F	5′-GGG CCC GGA TCC AGG CAT TTG CAA GAA TTA CTC-3′	*Bam*HI
PK002R	5′-GGG CCC GTC GAC TGT TTT ATA TTT GTT GTA AAA AGT AG-3′	*Sal*I

of the ATG start codon of *GPD1* and CGPD1D contains nucleotides 1,460 to 1,441 of the complementary strand downstream of the ATG start codon of *GPD1*. Subsequently, the *URA3* marker was looped out by homologous recombination of the two direct repeat fragments R on 5-FOA medium. The *gpd1*Δ mutant strain was denoted KAM-4 (*Mat* α*ura3 gpd1*Δ::REPEAT).

Over-expression of GLT1

An integration plasmid having both *PGK1* promoter and the coding sequence of the *GLT1* gene was constructed. First, the coding sequence of *GLT1* devoid of part of its promoter sequence was cloned by PCR with primers GT001F and GT002R and inserted to *Sal*I and *Pst*I sites of the plasmid YIplac211, resulting in the plasmid YIplac211-*GLT1*-tru. To loop out the *URA3* marker after integration, part of the *GLT1* promoter was inserted next to the *URA3* gene by cloning *Kpn*I and *Bam*HI-treated PCR product (amplified by primers GTP01F and GTP02R) to YIplac211-*GLT1*-tru previously cut with *Kpn*I and *Bam*HI. The resulting plasmid YIplac211-*GLT1*-tru-*GLT1*-pro was then digested with *Sal*I and *Bam*H I and ligated to the *Sal*I and *Bam*HI-treated PCR fragment containing *PGK1* promoter. Primers PK001F and PK002R were used in PCR to obtain the *PGK1* promoter sequence. The resulting plasmid was denoted YIplac211-P_{PGK1}-*GLT1*.

To integrate P_{PGK1}-*GLT1* to the genome, YIplac211-P_{PGK1}-*GLT1* was digested by *Bgl*II and the linearized plasmid was used to transform KAM-4. Cells were plated onto CM minus uracil medium plates. Correct insertion of the plasmid into the *GLT1* locus on chromosome was verified by PCR with primers GTP01F and GTP02R. Loop-out of the *URA3* marker gene by homologous recombination of the two direct *GLT1* promoter sequence was obtained by cultivating the correct transformations on 5-FOA plates and verified by PCR with primers GTP01F and GTP02R. The resulting mutant strain containing the P_{PGK1}-*GLT1* in place of the endogenous *GLT1* was denoted KAM-12 (*MAT*α *ura3 gpd1*Δ::REPEAT P_{PGK}-*GLT1*).

Fermentation conditions

All batch fermentation cultivations were carried out in 250 ml, cap-covered flasks with a working volume of 150 ml. Inoculum was cultured in YEPD medium until $OD_{600\ nm}$=1.0 in 100 ml Erlenmeyer flasks at 30°C, then 10% (*v*/*v*) preculture was transferred to the flasks with the same OD. The fermentation medium contains 160 g glucose l^{-1}, prepared from corn powder by the double-enzyme hydrolyzed procedure, 0.5 g $(NH_4)_2HPO_4$ l^{-1}, 0.3 g K_2HPO_4 l^{-1} added in the medium. During fermentations, the flasks were kept at 32°C in a thermostate chamber, and no air was supplied. Magnetic agitation rate was kept at 100 rpm, and pH 5 was kept by controlled addition of 2 mol NaOH l^{-1}.

Measurement of glucose, glycerol, ethanol, acetate, pyruvic acid, and protein contents

Two samples were centrifuged at 15,000 g for 8 min and the supernatant was frozen under −20°C until analysis. Pellet was washed twice with 0.9% (*w*/*v*) NaCl solution used to determine total protein by a modified biuret method. The concentrations of glucose, glycerol, ethanol, and pyruvic acid were determined by high performance liquid chromatography (HPLC) (HP1100, Japan) analysis. XDB-C8 column (Agilent, America) for determination of ethanol eluted with 0.25 mM H_2SO_4 with pH 3.1 at 30°C; ZORBAX carbohydrate column (Agilent) for determination of glycerol and glucose eluted with 75% acetonitrile at 30°C; RP18 column (Waters, Japan) for determination of pyruvic acid eluted with 0.1 mol KH_2PO_4 l^{-1} with pH 3.0 at 30°C. The flow rates were all of 1 ml min^{-1}. Detection was performed by means of differential refractive index detector. Acetate content was determined on gas chromatograph (Shimadzu, Japan) analysis with DB-WAX capillary column (Shimadzu). N_2 was used as a carrier gas and H_2 as a flaming gas with both at a flow rate of 32 ml min^{-1}.

Results

Growth characteristics

The strain KAM-4 and KAM-12, along with wild type strain KAM-2, were characterized with respects to several parameters including biomass and protein content. Similar growth rate was observed for KAM-4 and KAM-12 (Table 2). This result was further supported by the fact that the consumption of glucose in the two strains was basically indistinguishable (Fig. 1; Table 2). Similarly, the biomass and protein content in all three strains remained virtually unchanged (Table 2).

Table 2 Comparison of growth and compound yields *S. cerevisiae* wild type, KAM-4 (*MAT*α *ura3 gpd1*Δ::REPEAT) and KAM-12 (*MAT*α *ura3 gpd1*Δ::REPEAT P_{PGK}-*GLT1*) in batch fermentations

Parameters	KAM-2	KAM-4	KAM-12
Growth rate (h^{-1})	0.32±0.06	0.26±0.05	0.30±0.05
Biomass concentration (g/100 ml)	0.864± 0.048	0.863± 0.046	0.866± 0.045
Glycerol yield (g/100 ml)	0.44± 0.032	0.35± 0.020	0.32± 0.024
Ethanol (g/100 ml)	7.23±0.26	7.58±0.20	8.02±0.24
Protein (g/100 g biomass)	57.46± 0.45	56.54± 0.45	57.72± 0.44

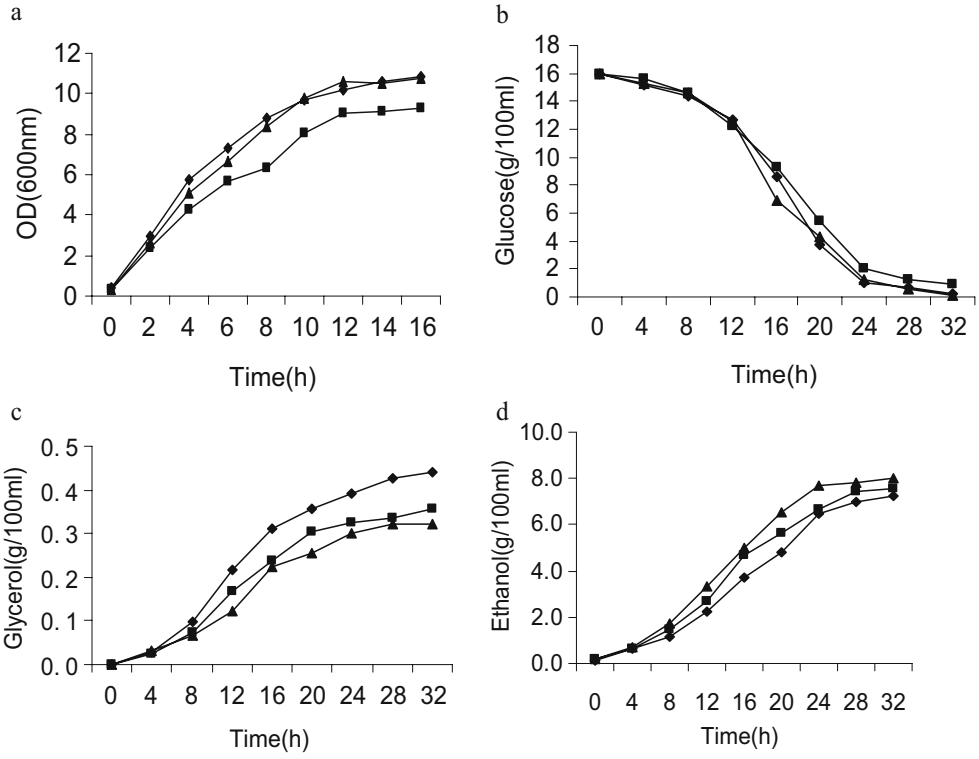

Fig. 1 Changes in measured parameters of OD of the strains, consumption of glucose, production of ethanol, and formation of glycerol during batch fermentations of wild type (*filled diamond* ♦), KAM-4 (*MATα ura3 gpd1*Δ::REPEAT) (*filled square* ■), and KAM-12 (*MATα ura3 gpd1*Δ::REPEAT P_{PGK}-*GLT1*) (*filled triangle* ▲), respectively, with 16% glucose as carbon source

Taken together, these results suggest that disruption of *GPD1* and integration of *GLT1* did not dramatically alter the cellular metabolic and biosynthetic pathways.

Analysis of fermentation products of the genetically engineered strains

During anaerobic batch fermentations, deletion of *GPD1* resulted in a decline in glycerol formation by 21.0% and by 25.0% in KAM-4 and KAM-12, respectively, compared to that of the wild type (Fig. 1). The ethanol production in both KAM-4 and KAM-12 was increased 4.8 and 10.8%, respectively, compared with that in the wild type at the end of fermentation (Fig. 1). Overexpression of *GLT1* in the *gpd1*Δ strain was able to produce more ethanol and form much less glycerol during batch fermentation on 16% (*w*/*v*) glucose compared with the wild type KAM-2. Besides that, dramatic decreasing of acetate and pyruvic acid formation was observed in strain KAM-4 and KAM-12 compared to the parental strain (Fig. 2).

Discussion

Deletion of *GPD1* and overexpressing *GLT1* resulted in an increase in ethanol production and a decrease in glycerol formation. This could be explained that the substrate that

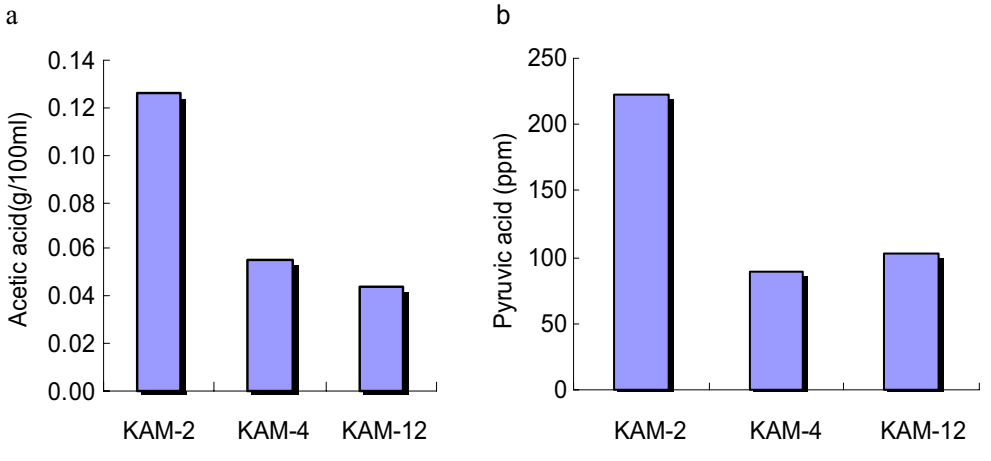

Fig. 2 Changes in measured parameters of acetate and pyruvic acid formation during anaerobic batch fermentations of wild type, KAM-4 (*MATα ura3 gpd1*Δ::REPEAT) and KAM-12 (*MATα ura3 gpd1*Δ::REPEAT P_{PGK}-*GLT1*), respectively, with 16% glucose as carbon source

would be used to form glycerol has been used to produce more ethanol. Although the metabolic pathway of ethanol production from glucose is redox neutral, the formation of certain metabolites leads to surplus production of NADH (Nissen et al. 2000). To prevent metabolism from inhibition, the surplus NADH formed in catabolic reactions needs to be reoxidized to NAD^+. Under aerobic conditions, this occurs by mitochondrial respiration, while under anaerobic conditions, the electron transport chain is not active and the necessary reoxidation of cytosolic NADH occurs by NADH-coupled reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (De Vries et al. 1992).

Theoretically, the effective way of solving the redox problem in the yeast cells is that the biomass would be made to contain less protein (Björkqvist et al. 1997). However, the decrease in glycerol formation was not accompanied by any decline in the protein content of the cells (Table 2). The results from this study showed that the cells were able to cope and maintain redox balance under anaerobic conditions even if glycerol formation was substantially decreased as observed in *gpd1*Δ mutants. The decrease drastically in acetate and pyruvic acid formation might be an example of a metabolic adjustment by the cells to minimize the NADH surplus when the glycerol production capacity is hampered (Fig. 2) (Valadi et al. 1998). In addition to *GPD1* deletion, overexpressing *GLT1* in the *gpd1*Δ mutant is possible to convert NADH to NAD^+ partly in the synthesis of glutamate from ammonium and α-oxoglutarate and resulting in a decrease in surplus formation of NADH and a lower glycerol production. Besides that, there must be additional mechanisms in the yeast cells as well. One possibility might be that *S. cerevisiae* has the potential use of other reductive pathways apart from glycerol formation during growth on glucose (Torben et al. 2000).

In summary, the present work has demonstrated the proposed concept to increase the ethanol production by deleting *GPD1* and overexpressing *GLT1* in *S. cerecisiae* minimizing glycerol formation under anaerobic conditions. In addition, formation of acetate and pyruvic acid was also reduced, which, conceivably, partially helped KAM-12 (*MAT*α *ura3 gpd1*Δ::REPEAT P_{PGK}-*GLT1*) cells manage to maintain redox balance in spite of the drastic reduction in glycerol level.

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