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ORIGINAL RESEARCH PAPER

Improved production of ethanol by deleting *FPS1* and over-expressing *GLT1* in *Saccharomyces cerevisiae*

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Abstract To improve ethanol production in *Saccharomyces cerevisiae*, two yeast strains were constructed. In the mutant KAM-3, the *FPS1* gene, which encodes a channel protein responsible for glycerol export, was deleted. The mutant KAM-11 had the *GLT1* gene (encoding glutamate synthase) placed under the *PGK1* promoter while having the *FPS1* deletion. Growth rate and biomass concentration remained virtually unchanged with the mutant KAM-11, compared to that of the parent. Over-expression of *GLT1* by the *PGK1* promoter along with *FPS1* deletion resulted in a 14% higher ethanol production and a 30% lower glycerol formation compared to the parental strain under anaerobic fermentation conditions. Furthermore, acetate and pyruvic acid formation was also reduced in order for cells to maintain redox balance.

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Introduction

Ethanol has become an alternative fuel to alleviate the high demand for gasoline. One of the many means of increasing ethanol production is to improve the ethanol-producing yield of yeast strains of *Saccharomyces cerevisiae*. Approaches have included the decreasing and/or elimination of yeast secondary metabolic products: for example, during the production of ethanol by *S. cerevisiae*, glycerol is produced at up to 5 g/l, which accounts for up to 5% of the carbon source in industrial fermentations (Nissen et al. 2000). Therefore, decreasing glycerol formation should increase the efficiency of ethanol production.

Glycerol formation plays role in maintaining cytosolic redox balance (Bakker et al. 2001) and is also involved in the osmoregulation of *S. cerevisiae* (Hohmann 2002). Ethanol production is redox-neutral, but the other cellular processes produce surplus cytosolic NADH, which is reoxidized by the glycerol 3-phosphate dehydrogenase (Gpd1p) to avoid an imbalance in the NAD^+/NADH ratio (Pahlman et al. 2001; Remize et al. 2001). One mean of decreasing excess NADH in yeast is the formation of glutamate, which

involves two coupled reactions, catalyzed by the NADH-dependent glutamate synthase (encoded by *GLT1* gene) and glutamine synthetase (encoded by *GLN1* gene) (Nissen et al. 2000). Overexpression of *GLT1* could therefore increase the conversion of NADH to NAD^+. This could in turn decrease the role of Gpd1p to carry out NADH reoxidized reaction, thus leading to a lower glycerol production (Nissen et al. 2000). On the other hand, decreasing glycerol formation could be achieved by deleting *FPS1*, which encodes a channel protein localized in the plasma membrane in *S. cerevisiae* (Hedfalk et al. 2004). Fps1p functions to mediate facilitated diffusion of glycerol to the outside of cells (Oliveira et al. 2003).

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 over-expressing *GLT1* in *fps1*Δ of *S. cerevisiae* (Michnick et al. 1997; Remize et al. 1999; Oner et al. 2005). Hence, in this work, we sought to genetic engineer yeast strains of *S. cerevisiae* with improved ethanol production via decreasing of glycerol formation by deleting *FPS1* and over-expressing *GLT1* (Fig. 1).

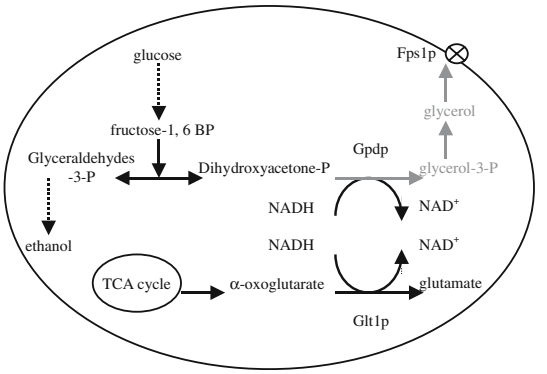

Fig. 1 Schematic representation of the ethanol metabolism in the KAM-11 (*MATα ura3 fps1*Δ::REPEAT P_{PGK}-*GLT1*), Gpdp, the glycerol 3-phosphate dehydrogenase; Glt1p, glutamate synthase

Materials and methods

Yeast strains and media

Saccharomyces cerevisiae haploid strain KAM-2 (*MATα ura3*) used in this study was derived from the diploid industrial strains TH-AADY (Angel Yeast Co., Ltd., China). Strains KAM-3 (*MATα ura3 fps1*Δ::REPEAT) and KAM-11 (*MATα ura3 fps1*Δ::REPEAT P_{PGK}-*GLT1*) were constructed in this study. 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).

One-step disruption of *FPS1*

To make the disruption cassette, five plasmids were sequentially constructed. The first plasmid pUC18-R was created by inserted a 446 bp of chromosomal DNA from *B. subtilis* 168 to pUC18. The 446 bp fragment was amplified by 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, pUC18-FRYURF was constructed where the RYUR cassette was flanked by portions of the start codon and of the stop codon of *FPS1* necessary for homologous recombination. First, the *FPS1* gene was amplified with primers F0011F and F0012R and cloned to the *Eco*RI and *Hind*III sites of pUC18, yielding pUC18-*FPS1*-ORF. Then, primers F0013F and F0014R were used to amplify a region of pUC18-*FPS1*-ORF such that both ends of the PCR product contain 130 bp and 120 bp of *FPS1*, respectively. Lastly, the PCR product was treated with *Pst*I and *Bam*HI, and ligated to the RYUR cassette previously excised from pUC18-RYUR by digestion

Table 1 List of primers used in this study

Primer name	Primer sequences	Restriction sites
R0011F	5′-GGG CCC GGA TCC GAG CAG CAT AAA CGA CTG CT-3′	*Bam*HI
R0012R	5′-GGG CCC TCT AGA ACG CTC AAT GTT GTT CAT GA-3′	*Xba*I
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
F0011F	5′-CCC GGG GAA TTC TAG TAG GAG AGC AGA GTG TC-3′	*Eco*RI
F0012R	5′-TAT AGT AGG TGA CCA GGC TG-3′	None
F0013F	5′-ATT TTT CTG CAG TGA GAA AAC AGA CAA GAA AAA GA -3′	*Pst*I
F0014R	5′-AGG CTG TCT AGA TGC ATT AGA ATG TAC CCT CG -3′	None
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

with *Pst*I and *Bam*HI to generate pUC18-FRYURF. The plasmid pUC18-FRYURF was linearized with *Eco*RI and *Hind*III and transformed into the wild type strain KAM-2. After growing on CM minus uracil for 2–3 days, transformants containing correct deletion of *FPS1* were verified by PCR with primers F0011F and F0012R. Subsequently, the *URA3* marker was looped out by via homologous recombination of the two direct repeat fragments R on 5-FOA medium. The *fps1*Δ mutant strain was denoted KAM-3.

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 order 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*HI 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-3. 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-11.

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

The concentrations of glucose, glycerol, ethanol and pyruvic acid were determined by HPLC. XDB-C8 and ZORBAX carbohydrate column (Agilent, USA) were used for the determination of ethanol, glycerol and glucose. An RP18 column was used for determination of pyruvic acid. The acetate content was determined by GC using a DB-WAX capillary column.

Results and discussion

Strain characterization

The two strain constructs KAM-3 and KAM-11, along with its parental strain KAM-2, were characterized with respects to several parameters including biomass and protein content. Similar growth rates were observed for KAM-2 and KAM-11 (Table 2). This result was further suported by the fact that the consumption of glucose in the two strains was basically indistinguishable (Fig. 2A). Similarly, the biomass and protein content in all three strains remained virtually unchanged (Table 2). Taken together, these results suggest that disruption of *FPS1* and/or integration of *GLT1* did not dramatically alter the cellular metabolic and biosynthetic pathways.

Analysis of fermentation products of the genetically engineered strains

There was dramatic accumulation of intracellular glycerol concentration inside the two mutant cells whose *FPS1* were deleted in comparison to the wild type (Fig. 2C), resulting in the glycerol amount excreted outside of the cells was reduced by 24% and 30% in strains KAM-3 and KAM-11, respectively, compared to that of the parental strain KAM-2 (Fig. 2B and Table 2). The two mutants, KAM-3 and KAM-11, had increased ethanol production by 10% and 14%, respectively, compared to the parental strain KAM-2

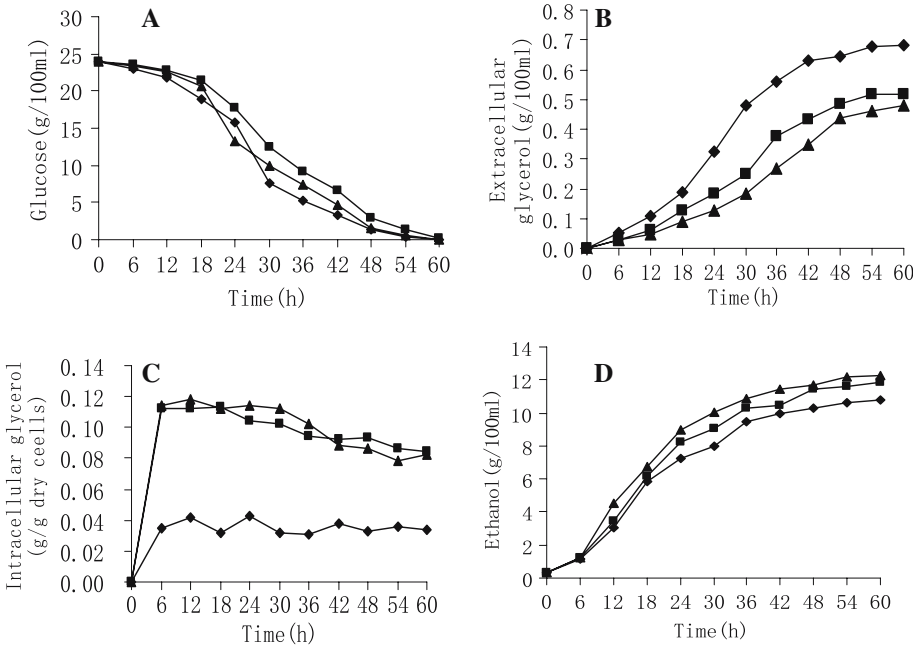

Fig. 2 Changes in measured parameters of glucose consumption, ethanol production and glycerol formation during batch fermentations of *S. cerevisiae* KAM-2 (*MATα ura3*) (♦), KAM-3 (*MATα ura3 fps1*Δ::REPEAT) (■) and KAM-11 (*MATα ura3 fps1*Δ::REPEAT P_{PGK}-*GLT1*) (▲), respectively, with 24% (g/v) glucose as carbon source. All batch fermentation cultivations were carried out in a 250 ml, cap-covered, flask with a working volume of 150 ml for three times. Cells was cultured in 100 ml Erlenmeyer flasks at 30°C in YEPD medium until $OD_{600\ nm}$ = 1.0. Then, 10% (v/v) of the culture was transferred to flasks containing fermentation medium prepared from the double-enzyme hydrolyzed corn powder with 240 g glucose/l. The flasks were kept at 32°C in a thermostatic chamber. Agitation rate was kept at 100 rpm, and pH 5 was kept. Cells were centrifuged at 15,000*g* for 8 min and the supernatant was used for analysis. Pellet was washed twice with 0.9% (w/v) NaCl solution. Part of the pellet was used to determine total protein, and the rest was diluted to a dry-weight concentration of 3 g/l with 0.9% NaCl and broken with glass beads for 20 min. Cell debris was removed by centrifugation and the supernatant was used for measuring the content of intracellular glycerol

Table 2 Comparison of growth and compound yields of KAM-2 (*MATα ura3*), KAM-3 (*MATα ura3 fps1*Δ::REPEAT) and KAM-11 (*MATα ura3 fps1*Δ::REPEAT P_{PGK}-*GLT1*) in batch fermentations

Parameters	KAM-2	KAM-3	KAM-11
Growth rate (h^{-1})	0.28	0.22	0.26
Biomass concentration (g/100 ml)	0.86	0.86	0.87
Protein content (g/100 g biomass)	58	58	58
Glycerol yield (g/100 ml)	0.68	0.52	0.48
Glycerol yield (mol glycerol/mol glucose)	0.028	0.022	0.020
Ethanol (g/100 ml)	10.8	11.9	12.3
Ethanol yield (mol ethanol/mol glucose)	0.47	0.50	0.51

(Fig. 2D and Table 2). Therefore, we demonstrated that deletion of *FPS1* enhanced ethanol production and decreased glycerol formation. Furthermore, over-expression of *GLT1* in the *fps1*Δ background increased the capacity to produce even more ethanol while maintaining the decreased ability to form glycerol. Besides that, a large decrease of acetate and pyruvic acid formation was observed in strains KAM-3 and KAM-11 compared to the parental strain (Fig. 3A, B).

Deletion of *FPS1* and over-expression of *GLT1* resulted in increased ethanol production and decreased glycerol formation. Presumably, the substrate that would have been used to form glycerol was redirected for ethanol production. Unlike wild type yeast cells that decrease their intracellular glycerol content quickly when adapting to hypo-osmotic shock, the *fps1*Δ mutants requires more time to achieve the same glycerol reduction (Fig. 2C). Consequently the glycerol accumulation in *fps1*Δ mutants may cause the feedback inhibition of the metabolic pathways related to the formation of glycerol, resulting in overall decrease of glycerol amount formed in cells. This is an example of a metabolic adjustment by cells when the glycerol production capacity is hampered. The drastic decrease in acetate and pyruvic acid formation might be another example of a metabolic adjustment (Valadi et al. 1998). Theoretically, the effective way of solving the redox problem in the yeast cells is that less protein would be made in the biomass (Björkqvist et al. 1997). However, the decrease in glycerol formation was not accompanied by any decline in the protein content of the biomass (Table 2). Such results indicated that the cells were able to cope and maintain redox balance under anaerobic conditions even if glycerol formation was substantially decreased in *fps1*Δ mutants. Over-expression of *GLT1* in *fps1*Δ mutant was also able to partially convert NADH to NAD^+ in the synthesis of glutamate from ammonium and α-oxoglutarate, resulting in a lower glycerol formation. Lastly, other reductive pathways apart from glycerol formation during fermentation on glucose should exist in the yeast cells to concertedly cope with redox balancing (Nissen et al. 2000).

In conclusion, the present work has demonstrated the proposed concept to increase the ethanol production by deleting *FPS1* and

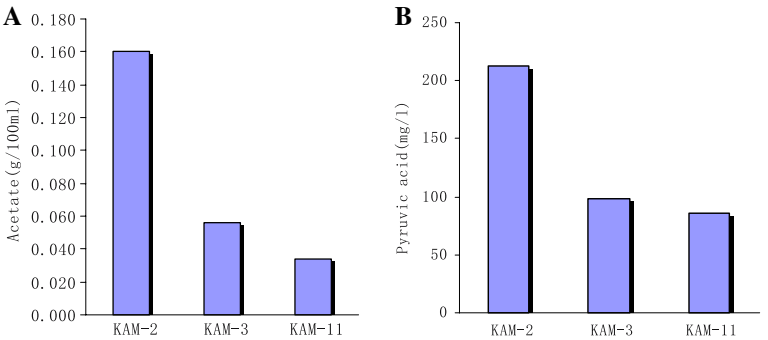

Fig. 3 Changes in measured parameters of acetate and pyruvic acid formation during anaerobic batch fermentations of *S. cerevisiae* KAM-2 (*MATα ura3*), KAM-3 (*MATα ura3 fps1*Δ::REPEAT) and KAM-11 (*MATα ura3 fps1*Δ::REPEAT P_{PGK}-*GLT1*), respectively, with 24% (g/v) glucose as carbon source

over-expressing *GLT1* in *S. cerevisiae*. This was achieved by closing up the glycerol efflux channel and thus minimizing glycerol formation under anaerobic conditions. In addition, formation of acetate and pyruvic acid was also reduced, which, conceivably, partially helped *fps1Δ* P_{PGK1}-*GLT1* mutant (KAM-11) cells manage to maintain redox balance in spite of the drastic reduction in glycerol level.

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