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Deletion of the *CgTPI* Gene Encoding Triose Phosphate Isomerase of *Candida glycerinogenes* Inhibits the Biosynthesis of Glycerol

Zhang Yongguang, Shen Wei, Rao Zhiming, Fang Huiying, Zhuge Jian

The Key Lab of Industrial Biotechnology of Ministry of Education, Research Centre of Industrial Microorganisms, School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China

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Abstract. The yeast *Candida glycerinogenes* produces a high yield of glycerol only in response to a medium-osmotic stress, but little is known about the relationship between osmoadaptation and glycerol metabolism. The *CgTPI* gene encoding triose phosphate isomerase of *C. glycerinogenes* was cloned and sequenced, and its functionality was confirmed by complementation of *Saccharomyces cerevisiae tpi1* Δ. The roles of CgTpip in the glycerol biosynthesis and the osmoadaptation were investigated. Unlike *S. cerevisiae tpi1* Δ and *Klyuveromyces lactis tpi1* Δ, the mutant lacking *CgTPI* significantly decreased the rate of glucose consumption and the glycerol yield. Furthermore, the mutants decreased osmotolerance to glucose and NaCl. The results suggest that *CgTPI* might be crucial for a high yield of glycerol by *C. glycerinogenes*. The inhibition of glycerol biosynthesis might be related to the reduced ability of osmoadaptation to high external osmolarity. To our knowledge, this is the first report that inactivation of a yeast *TPI* gene inhibits the biosynthesis of glycerol.

The accumulation of osmoprotective solutes such as glycerol, arabitol, mannitol, and erythritol, is an ubiquitous strategy of cellular osmoadaptation in yeast [25]. In most yeast species, glycerol is the main osmolyte that is produced and accumulated intracellularly in response to hyperosmotic stress. The physiological function suggests that osmotolerant yeasts are of particular interest for glycerol production [1, 3, 24]. Strategies such as modification of NADH production and consumption and direct influence of the carbon flux have been used to increase glycerol yield from glucose by *Saccharomyces cerevisiae* [1, 22, 24]. For example, deletion of *TPI1* gene encoding triose phosphate isomerase will result in an increase of glycerol yield in *S. cerevisiae* [7].

Triose phosphate isomerase catalyzes the reversible interconversion of glyceraldehydes 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), which fall into important metabolic branch points between glycolysis, gluconeogenesis, the pentose pathway, glycerol production/utilization, and the methylglyoxal pathway [10, 11]. The genes encoding triose phosphate isomerase have been cloned from several yeast strains [2, 8, 15]. In *S. cerevisiae* and *Klyuveromyces lactis*, the lack of TIM activity causes an accumulation of DHAP, which leads to a redirection of the NADH reoxidation, and enhances glycerol yield [7, 8, 17].

Candida glycerinogenes, an osmotolerant yeast isolated from southern China, is currently used commercially in China for the production of glycerol because of its high yield, productivity, and recovery yield [24, 26]. Distinct from other osmotolerant yeast, its yield of glycerol on glucose is beyond the theoretical value (1 mol/mol). This peculiar feature suggests that some other pathways might be involved in glycerol biosynthesis in addition to glycolysis. Therefore, research on glycerol metabolism and the interaction of metabolic pathways would be valuable and practical. Based on the mechanisms that the hexose monophosphate pathway (HMP) plays an important role in polyol biosynthesis in osmotolerant yeast exposed to high osmotic stress [20, 21], it is speculated that triose phosphate isomerase (TIM) might participate in glycerol biosynthesis of *C. glycerinogenes*, by which GAP formed from HMP is catalyzed by the triose phosphate isomerase activity. In this study, we

Correspondence to: Z. Jian; *email:* jianzhuge@hotmail.com

aimed to clone the *C. glycerinogenes* gene encoding TIM and to test its role in glycerol biosynthesis. Conversely to the *S. cerevisiae* and *K. lactis* mutants, the deletion of *CgTPI* decreased the glycerol yield and productivity and also the ability of osmoadaptaton to high external osmolarity.

Materials and Methods

Yeast Strains and Growth Conditions. *Candida glycerinogenes* WL2002-5, from the Research and Design Center of Glycerol Fermentation, Southern Yangtze University [26], was grown in YPD medium (yeast extract (1% w/v), bacto-peptone (2% w/v), glucose (2% w/v), and agarose (1.5% w/v) for solid medium). *Saccharomyces cerevisiae* W303-1A (*MATα, leu2-3, ura3-1, trp1-1, his3-11, ade2-1, can1-100*) was used for the construction of *S. cerevisiae tpi1* Δ. Difco yeast nitrogen base (YNB) without amino acid, supplemented with 50 μg/mL of the required supplements, and carbon sources were used for growth on minimal selective media. As necessary, 150 μg Zeocin/mL or 500 μg G418/mL were added. *Escherichia coli* DH5α was cultured in Luria–Bertani (LB) medium supplemented with 100 μg ampicillin/mL or 25 μg Zeocin/mL and used for plasmid propagation.

Liquid growth assays were performed by pregrowing the strains in YNBG medium (0.67% YNB, 2% glucose, w/v). Then cultures were inoculated at 5% (v/v) into media containing 0.67% (w/v) YNB with 10, 50, 100, 200, 300, 400, 500, or 600 g glucose/L^1, or 2% glucose with 0.2, 0.5, 1.0, 1.5, or 2.0 M NaCl. Cell density was monitored by determining the optical density at 600 nm (OD_{600}) after inoculation for 24 h and 48 h, respectively. Shake-flask cultivations were performed following Zhuge et al. [26] except that the fermentation medium was composed of 210 g/L glucose, 2 g/L urea, 0.4 g/L NaCl, 0.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L $CaCl_2$ 0.1.

Isolation and Sequencing of the *CgTPI* Gene. Genomic DNA of *C. glycerinogenes* was extracted as described previously [5]. On the basis of sequence similarity between yeast triose phosphate isomerases, two degenerate oligonucleotides were designed—1F: 5′-GC(TC) TAC GA(AG) CC(AT) GT(CTG) TGG GC-3′, encoding AYEPVWA [amino acid residues 166–172]; 1R: 5′-GCA CCA CC(AG) AC (ATCG) A(AG)G AAA CC-3′, encoding GFLVGGA [amino acid residues 231–237]. The amplification procedure consisted of an initial denaturation and enzyme activation step at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and one final extension at 72°C for 6 min. A polymerase chain reaction (PCR) product of 215 bp was cloned using the pMD18-T vector cloning kit (TaKaRa Co.) and then sequenced. Based on the known fragment, inverse PCR was performed as described previously [16]. The primers were as follows: 2F [corresponding to the open reading frame (ORF) nucleic acid 611–632]: 5′-CCG TAT AGG ATT CTG ACA GCT T; 2R (corresponding to the ORF nucleic acid 651–672): 5′-GGA TGC ACC AGA ATT CAA GGA C-3′. The same PCR procedure described earlier had been used.

Complementation of *S. cerevisiae tpi1* Δ. The *S. cerevisiae tpi1* Δ was constructed by the PCR-mediated direct gene disruption method [14]. A deletion extending from the site (+128) to the site (+ 421) inside the ORF of *TPI1* in *S. cerevisiae* strain W303-1A was replaced by the kanMX module containing the kanamycin-resistance ORF of pUG6 [13], conferring on yeast resistance against G418. Gene disruption was tested by the assay of the triose phosphate isomerase in the ura^+ transformants that did not grow on the minimal medium containing 2% glucose (w/v). *CgTPI* with its own promoter was introduced into the *Nco*I site of pYX212 (kindly supplied by Bernard A. Prior) and then transferred into the *S. cerevisiae tpi1* Δ by the lithium acetate transformation method [12].

Construction of *CgTPI* Deletion Mutant. A deletion extending from the site (+203) to the site (+ 469) inside the ORF of *CgTPI* in the wild-type strain of *C. glycerinogenes* WL-2002-5 was replaced by the *Zeocin* module containing the *Zeocin*-resistance ORF of pGAPZ B (Invitrogen Co.) [18]. Then one-step gene disruption method was used [19]. Stable Zeocin-resistant transformants were selected on a YPD-agar plate containing 150 μg Zeocin/mL and confirmed by the assay of triose phosphate isomerase activity and Southern analysis. DIG High Prime DNA labeling and Detection Kit I (Boehringer Mannheim Gmbh, Mannheim, Germany) was used according to the supplier's manual.

Enzyme Assay. Cell extracts were prepared as described previously [23]. The protein concentration of cell-free extract was determined as the method of Bradford [4] by using bovine serum albumin as the standard. The specific activity of the triose phosphate isomerase was determined on cell extracts according to Compagno et al. [7].

Determination of DHAP, Glucose, and Glycerol. Determination of intracellular DHAP was performed as described previously [7]. Glucose was determined by immobilized glucose oxidase using a glucose analyzer. Glycerol was monitored as described previously [26].

Results

Cloning and Sequence Analysis of the *CgTPI C. glycerinogenes* WL2002-5. A 1388-bp fragment was obtained from *C. glycerinogenes* WL2002-5 by using the degenerate PCR and the inverse PCR. The sequence analysis revealed a 753-bp ORF, encoding a protein of 250 deduced amino acids with the predicted molecular weight of 26,729 Daltons. The amino acid sequence was compared to other yeast TIM reported in GenBank using the DANMAN 5.0 program and confirmed that the protein was a member of yeast TIM. It showed 70.0%, 68.33%, and 68.75% homology to *S. cerevisiae* Tpip, *K. lactis* Tpip, and *Zygosaccharomyces bailii* Tpip, respectively. Therefore, the gene was designated as *CgTPI*. Compared with other yeast genes encoding TIM reported in GenBank, the nucleic acid sequence of the *CgTPI* exhibited 70.94%, 68.08%, 67.12%, 67.8%, 68.76%, 64.96%, 51.45%, and 53.14% homology to *S. cerevisiae* (accession No. J01366), *Z. bailii* (accession No. AF325852), *K. lactis* (accession No. AJ012317), *Debaryomyces hansenii* (accession No. AL436697), *Candida glabrata* (accession No. XM_447161), *Candida albicans* (accession No. AF124845), *Cryptococcus neoformans* (accession No. XM_571026), and *Schizosaccharomyces pombe* (accession No. M14432), respectively. The *CgTPI* nucleotide sequence has been assigned the GenBank accession No. EF392679.

Functional Analysis of *CgTPI* in *S. cerevisiae tpi1* Δ Mutant. In order to test the function of *CgTPI* in *S. cerevisiae*, complementation analysis was performed

in the *S. cerevisiae tpi1* Δ constructed. The *S. cerevisiae tpi1* Δ mutant was selected and confirmed as described by Compagno et al. [7]. In a mutant strain lacking TIM, a net energy gain from glycolysis alone cannot be obtained, because only half of the glucose flows through the glycolytic pathway. Therefore, the *S. cerevisiae tpi1* Δ mutant was unable to grow on minimal YNB medium containing glucose as the sole carbon source and also lost TIM activity. When the plasmid pYX212-*CgTPI* constructed was transformed into and expressed in *S. cerevisiae tpi1* Δ, the cells harboring *CgTPI* were observed to grow on minimal YNB medium with glucose as the sole carbon resource and restore triose phosphate isomerase activity (11.5 ± 1.1units/mg protein). The results indicate that the *CgTPI* is functional in the *S. cerevisiae tpi1* Δ mutant.

Construction and Growth Properties of the *C. glyucerinogenes tpi* Δ Mutant Strain. To test the physiological importance of CgTpip in *C. glycerinogenes*, a CgTpip deletion mutant was constructed in the wild-type strain of *C. glycerinogenes* WL2002-5 by the one-step gene disruption method using the Zeocin-resistance module. In cell extracts of one clone, triose phosphate isomerase activity was not detectable; it was 12.5 ± 1.2 units/mg protein in the wild- type strain. The result suggests that disruption of the *CgTPI* gene had occurred and also that the right and functional CgTpip encoding gene was cloned. The size of the hybridizing band (2.3 kb) in the DNA of the mutant strain corresponded exactly with the expected size of the fragment, which was consistent with the size of the disrupted copy of the *CgTPI* gene. The lack of triose phosphate isomerase activity in cell extracts of this clone and the presence of only one hybridizing band in the DNA of the wild-type strain indicate that the gene encoding TIM in *C. glycerinogenes* is present in one copy.

The deletion of *CgTPI* would affect the metabolism of the mutant, which would be reflected by its phenotype, such as growth or osmotolerance. Therefore, the growth and osmotolerance of the *C. glyucerinogenes tpi* Δ mutant strain were tested in the present study. When cultured on the minimal YNB medium containing glucose as the sole carbon source, the *C. glycerinogenes tpi* Δ mutant strain was able to grow, as reported in the *K. lactis tpi1* Δ mutant [8]. Compared with the wild-type strain, the *C. glycerinogenes tpi* Δ mutant altered its osmotolerance to high external osmolarity. The *C. glycerinogenes tpi* Δ mutant decreased the optimum glucose concentration for growth (Figs. 1A and 1B) and also the osmotolerance to NaCl (Figs. 1C and 1D). These observations indicate that the mutant strain is osmosensitive to high NaCl and glucose; the inactivation of triose phosphate isomerase activity decreases the tolerant ability to high external osmolarity.

Deletion of *CgTPI* Inhibits the Biosynthesis of Glycerol. In order to easily analyze the metabolites and exclude the effects of natural organic materials, such as corn steep liquor, the chemical-defined fermentation medium was used. Compared to the wild-type strain, the glucose consumption was extremely reduced in the *C. glycerinogenes tpi* Δ mutant (Fig. 2A); this is consistent with the reports on *S. cerevisiae tpi1* Δ [7] and *K. lactis tpi1* Δ [8]. The average specific glucose consumption rate for the *C. glycerinogenes tpi* Δ mutant and wild-type strains were 0.092 h^{-1} and 0.156 h^{-1}, respectively (Table 1). The glycerol production and productivity were also decreased in the *C. glycerinogenes tpi* Δ mutant (Fig. 2B). The maximal glycerol and the average specific glycerol production rate for the *tpi* Δ mutant were 87.13 g/L and 0.039 h^{-1}, whereas for the wild-type strain, they were 108.41 g/L and 0.080 h^{-1}, respectively (Table 1). It is notable that the glycerol yield and the average specific glycerol production rate were markedly decreased in the *C. glycerinogenes tpi* Δ mutant strain, which were not consistent with the *S. cerevisiae tpi1* Δ and *K. lactis tpi1* Δ mutant strains [6–9]. Furthermore, the *C. glycerinogenes tpi* Δ mutant also decreased the glycerol yield on glucose consumed (g/g) from 0.524 (1.0346 mol glycerol/mol glucose) to 0.425 (0.8263 mol glycerol/mol glucose) (Table 1). Especially after 30 h of fermentation, the decrease phenomena were more clearly found in the *C. glycerinogenes tpi* Δ mutant (data not shown).

To test whether DHAP, the glycerol precursor, was accumulated in the *C. glycerinogenes tpi* Δ mutant, the intracellular DHAP and glycerol were determined. Contrary to the *S. cerevisiae tpi1* Δ and *K. lactis tpi1* Δ mutant strains [6, 7, 9], the *C. glycerinogenes tpi* Δ mutant accumulated less DHAP than the wild-type strain (Fig. 3). The intracellular glycerol of the *C. glycerinogenes tpi* Δ mutant was also less than that of the wild-type strain (data not shown). The result that less DHAP accumulated in the *C. glycerinogenes tpi* Δ mutant indicates that the deletion of *CgTPI* might reduce the content of functional triose phosphate for the biosynthesis of glycerol, which might be related to the decrease of glucose consumption.

Discussion

The strategy improving the availability of functional triose by deletion of the gene encoding triose phosphate isomerase has been proven successfully to enhance the

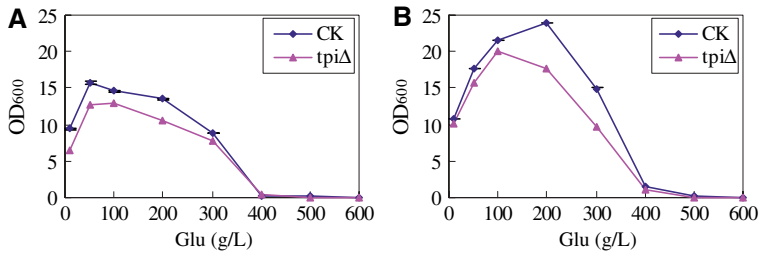

Fig. 1. Growth of the wild-type *C. glycerinogenes* (CK) and *C. glycerinogenes tpi* Δ (*tpi* Δ) mutant strains in YNB medium containing different concentrations of glucose for 24 h (**A**) or 48 h (**B**) and NaCl for 24 h (**C**) or 48 h (**D**), respectively. Results are represented as the means of three independent experiments with standard deviation.

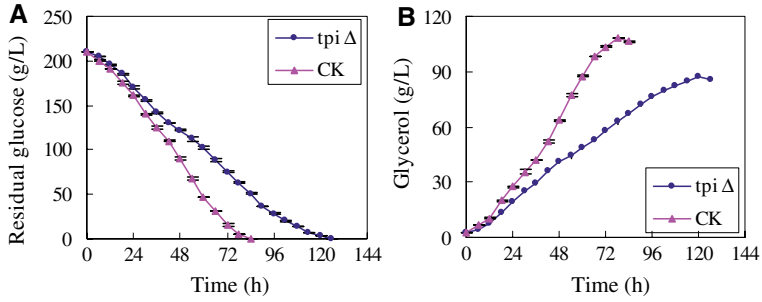

Fig. 2. The course of glucose consumption (**A**) and glycerol production (**B**) by the wild-type *C. glycerinogenes* (CK) and *C. glycerinogenes tpi* Δ (*tpi* Δ) mutant strains in chemical-defined fermentation medium. Results are represented as the means of two independent triplicate experiments with standard deviation.

Table 1. Glycerol fermentation parameters of main products in the *C. glycerinogenes tpi* Δ (*tpi* Δ) and the wild-type (CK) strains during cultivation in chemical-defined fermentation medium

Parameters	*tpi* Δ	CK
Initial glucose concentration (g/L)	210	210
Residual glucose concentration (g/L)	3.3	5
Fermentation time (h)	120	78
Maximum dry cell weight (g/L)	20.01	19.28
Maximum glycerol production (g/L)	87.13	108.41
Glucose consumption rate (g/L/h)	1.71	2.65
Average specific glucose consumption rate (h^{-1})	0.092	0.156
Average specific growth rate (h^{-1})	0.008	0.014
Average specific glycerol production rate (h^{-1})	0.039	0.080
Biomass yield on glucose (g/g)	0.093	0.087
Glycerol yield on glucose consumed (g/g)	0.425	0.524
Biomass productivity (g/L/h)	0.160	0.231
Glycerol productivity (g/L/h)	0.73	1.39

Note: The values reported are the means of two independent triplicate experiments determination with less than 5%.

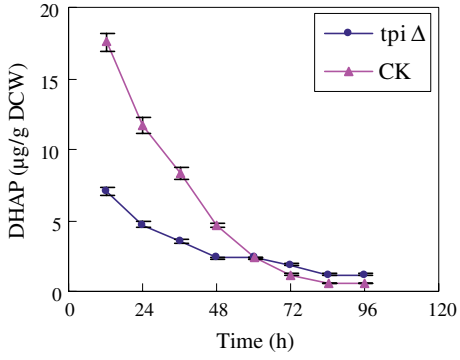

Fig. 3. Intracellular content of DHAP in the *C. glycerinogenes tpi* Δ (*tpi* Δ) and the wild-type *C. glycerinogenes* (CK) strains. Results are represented as the means of two independent triplicate experiments with standard deviation.

glycerol yield in *S. cerevisiae* [7, 9, 17] or *K. lactis* [6, 8]. At the same time, the null mutation of the gene also results in growth deficiencies, such as the inability to grow on glucose as the whole carbon source and sensitivity to high glucose concentration, as found in *S. cerevisaiae* [7, 9]. In this study, the gene encoding triose phosphate isomerase from *C. glycerinogenes* was cloned, and its role in glycerol formation was studied.

Our results showed that the deletion of *CgTPI* greatly decreased the concentration of the intracellular DHAP and the glycerol yield and productivity. The

results were opposite to previous reports on *S. cerevisiae tpi1* Δ [7, 9] and *K. lactis tpi1* Δ mutant strains [6]. The inhibition of glycerol biosynthesis might be related to the disparity of glycerol biosynthesis pathways [1], the $NADH/NAD^+$ equilibrium, and the reduced ability of osmoadaptation to high external osmolarity. One of the main advantages of osmotolerant yeast for glycerol production is higher sugar concentration in fermentation media, which can be used for an improved glycerol production rate and yield [1, 22, 24]. In this study, the decrease of osmotolerance is related to the decrease of glycerol yield, which means that the osmotolerance property is the base for *C. glycerinogens* to produce a high yield of glycerol. A notable observation of the *C. glycerinogenes tpi* Δ mutant strain is that the glycerol yield on glucose consumed was significantly decreased to 0.8263 (mol/mol), 20.13% lower than the wild-type strain (1.0346 mol/mol). The observation suggests that the *CgTPI* is essential for a high yield of glycerol; the deletion of *CgTPI* would inhibit the glycerol biosynthesis.

In conclusion, our results suggest that *CgTPI* might be crucial for a high yield of glycerol by *C. glycerinogenes*. The inhibition of glycerol biosynthesis might be related to the reduced ability of osmoadaptation to high external osmolarity. Further work is required to study the detailed relationship between glycerol metabolism and osmoadaptation.

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Literature Cited

1. Agarwal GP (1990) Glycerol. In: Fiechter A (eds.) Advances in biochemical engineering/ biotechnology: Microbial Bioproducts Vol. 41. Heidelberg: Springer-Verlag, pp 95–128
2. Alber T, Kawasaki G (1982) Nucleotide sequence of the triose phosphate isomerase gene of *Saccharomyces cerevisiae*. J Mol Appl Genet 1:419–434
3. Ansell R, Granath K, Hohmann S, et al. (1997) The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. Embo J 16:2179–2187
4. Bredford MM (1976) Rapid and sensitive method of the quantification of microgram quantities of protein utilizing the principal of protein-dye binding. Anal Biochem 72:248–254
5. Burke D, Dawson D, Stearns T (2000) Methods in yeast genetics, a Cold Spring Harbor Laboratory course manual. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 109–110
6. Capitanio D, Merico A, Ranzi BM, et al. (2002) Effects of the loss of triose phosphate isomerase activity on carbon metabolism in *Kluyveromyces lactis*. Res Microbiol 153:593–598
7. Compagno C, Boschi F, Ranzi BM (1996) Glycerol production in a triose phosphate isomerase deficient mutant of *Saccharomyces cerevisiae*. Biotechnol Prog 12:591–595
8. Compagno C, Boschi F, Daleffe A, et al. (1999) Isolation, nucleotide sequence, and physiological relevance of the gene encoding triose phosphate isomerase from *Kluyveromyces lactis*. Appl Environ Microbiol 65:4216–4219
9. Compagno C, Brambilla L, Capitanio D, et al. (2001) Alterations of the glucose metabolism in a triose phosphate isomerase-negative *Saccharomyces cerevisiae* mutant. Yeast 18:663–670
10. Cooper RA (1984) Metabolism of methylglyoxal in microorganisms. Annu Rev Microbiol 38:49–68
11. Gancedom C, Serrano R (1989) Energy-yielding metabolism. In: Rose AH, Harrison JS (eds.) The yeast, Vol. 3. London: Academic Press, pp 205–209
12. Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350:87–96
13. Güldener U, Heck S, Fielder T, et al. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24:2519–2524
14. Kaur R, Ingavale SS, Bachhawat AK (1997) PCR-mediated direct gene disruption in *Schizosaccharomyces pombe*. Nucleic Acids Res 25:1080–1081
15. Merico A, Rodrigues F, Corte-Real M, et al. (2001) Isolation and sequence analysis of the gene encoding triose phosphate isomerase from *Zygosaccharomyces bailii*. Yeast 18:775–780
16. Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623
17. Overkamp KM, Bakker BM, Kotter P, et al. (2002) Metabolic engineering of glycerol production in *Saccharomyces cerevisiae*. Appl Environ Microbiol 68:2814–2821
18. pGAPZ A, B, and C, pGAPZαA, B, and C, Pichia expression vectors for constitutive expression and purification of recombinant proteins. Version F, September 3, 2002, 25-0174. Source: Invitrogen Life Technologies. Available at: www.invitrogen.com
19. Rothstein RJ (1983) One-step gene disruption in yeast. Methods Enzymol 101:202–211
20. Spencer JF, Shu P (1957) Polyhydric alcohol production by osmophilic yeasts: effect of oxygen tension and inorganic phosphate concentration. Can J Microbiol 3:559–567
21. Spencer JF, Neish AC, Blackwood AC, Sallans HR (1956) Polyhydric alcohol production by osmophilic yeasts: studies with C^{14}-labeled glucose. Can J Biochem Physiol 34:495–501
22. Taherzadeh MJ, Adler L, Liden G (2002) Strategies for enhancing fermentative production of glycerol: a review. Enzyme Microb Technol 31:53–66
23. van Hoek P, van Dijken JP, Pronk JT (2000) Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme Microb Technol 26:724–736
24. Wang Z-X, Zhuge J, Fang H-Y, et al. (2001) Glycerol production by microbial fermentation: a review. Biotechnol Adv 19:201–223
25. Yancey PH, Clark ME, Hand SC, et al. (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–1222
26. Zhuge J, Fang H-Y, Wang Z-X, et al. (2001) Glycerol production by a novel osmotolerant yeast *Candida glycerinogenes*. Appl Microbiol Biotechnol 55:686–692