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Enhanced Production of 1,2-Propanediol by *tpi1* Deletion in *Saccharomyces cerevisiae*

Jung, Joon-Young, Eun-Sil Choi, and Min-Kyu Oh*

Department of Chemical and Biological Engineering, Korea University, Seoul 136-713, Korea

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***Saccharomyces cerevisiae* was metabolically engineered to improve 1,2-propanediol production. Deletion of the *tpi1* (triosephosphate isomerase) gene in *S. cerevisiae* increased the carbon flux to DHAP (dihydroxylacetone phosphate) in glycolysis, resulting in increased glycerol production. Then, the *mgs* and *gldA* genes, the products of which convert DHAP to 1,2-propanediol, were introduced to the *tpi1*-deficient strain using a multicopy plasmid. As expected, the intracellular level of methylglyoxal was increased by introduction of the *mgs* gene in *S. cerevisiae* and that of 1,2-propanediol by introduction of both the *mgs* and *gldA* genes. As a result, 1.11 g/l of 1,2-propanediol was achieved in flask culture.**

Keywords: *Saccharomyces cerevisiae*, triosephosphate isomerase, 1,2-propanediol, metabolic engineering

1,2-Propanediol, also known as propylene glycol, is a major commodity chemical for the food, drug, and cosmetic industries. It is used as less-toxic antifreeze, solvent in mixing chemicals in the photographic industries, solvent for coloring, flavors and humectants food additives in the food industries, and moisturizer, lubricants, and carrier of fragrance oils in the cosmetic and pharmaceutical fields [1]. The U.S. Food and Drug Administration (FDA) has determined propylene glycol to be "generally recognized as safe" for use in food, cosmetics, and medicines.

There have been a few reports to produce 1,2-propanediol from several microorganisms using the biochemical process. Two pathways have been used to produce 1,2-propanediol. First, deoxy sugars can be used as a carbon source to form lactaldehyde, which is subsequently converted to 1,2-propanediol. Badia *et al.* [5] and Lin [18] studied deoxy sugar metabolism in *Salmonella typhimurium* and *Escherichia coli* and developed a pathway to produce 1,2-propanediol from deoxy sugars. Forsberg and Gibbons [10] also produced *S*(+)-1,2-propanediol from lactaldehyde. However, this pathway is not commercially interesting owing to the high cost of deoxy sugars.

A second pathway is the conversion of DHAP (dihydroxyacetone phosphate) to 1,2-propanediol *via* methylglyoxal. The studies about this pathway identified the *mgs* (methylglyoxal synthase) and *gldA* (glycerol dehydrogenase) genes for 1,2-propanediol production. Using this pathway, Cameron and Cooney [7] produced 1,2-propanediol in *Clostridium thermosaccharolyticum*, as did Altaras and Cameron in *E. coli*, Huang *et al.* [12] in *Clostridium acetobutylicum*, and Lee and DaSilva [16] in *Saccharomyces cerevisiae*. The productivity ranged from 9.0 g/l with *Thermoanaerobacterium thermosaccharolyticum* to 0.49 g/l with *E. coli* and 0.52 g/l with *S. cerevisiae* [6]. Although a high concentration of 1,2-propanediol was produced from *C. sphenoides* or *T. thermosaccharolyticum*, their genome and physiology have not been well studied, meaning that metabolic engineering or gene cloning with those strains is very difficult. Meanwhile, the metabolic enzymes for producing 1,2-propanediol was well characterized in *E. coli*.

In this study, we produced 1,2-propanediol in metabolically engineered *S. cerevisiae* to improve the carbon flux to target metabolite. We chose *S. cerevisiae* because it is well studied, easy to manipulate, and known as a good host to produce glycerol that is also produced from DHAP. Three gene manipulations were conducted for improving 1,2-propanediol productivity. The first one was the deletion of *tpi1* (triosephosphate isomerase), which plays a role in interconversion between DHAP and GAP (glyceraldehyde-3-phosphate) (Fig. 1). The deletion was expected to increase the carbon flux to the DHAP side [9]. The next was the overexpression of *mgs* and *gldA*, which play roles in the syntheses of methylglyoxal and 1.2-propanediol, respectively. These genes do not exist in *S. cerevisiae*, so the *E. coli* genes were cloned and introduced to *S. cerevisiae*. Through these gene manipulations, a total of 1.11 g/l of 1,2-propanediol was produced in *S. cerevisiae* by a flask culture.

*Corresponding author
Phone: +82-2-3290-3308; Fax: +82-2-926-6102;
E-mail: mkoh@korea.ac.kr

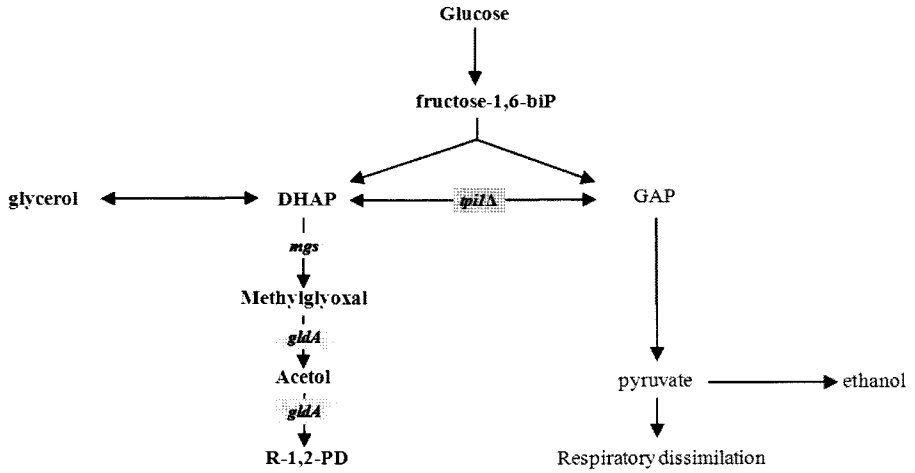

Fig. 1. Metabolic pathway for 1,2-propanediol production in *Saccharomyces cerevisiae*.
DHAP, GAP, and R-1,2-PD represent dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, and R-1,2-propanediol, respectively. The engineered genes, *tpi1*, *mgs*, and *gldA*, encode triosephosphate isomerase 1, methylglyoxal synthase, and glycerol dehydrogenase, respectively.

Materials and Methods

Strains and Plasmid Constructions

Y2805 (*MATa pep4::HIS3 prb1*-d *can1 GAL2 his3 ura3-52*) strain was genetically modified for producing 1,2-propanediol (Table 1). *E. coli* strain DH5α was used for plasmid construction.

To delete the *tpi1* gene, a homologous recombination method using the truncated *Kluyveromyces lactis URA3* (*Kl URA3*) gene in two vectors, pWAL200 and pWBR200, was used [17]. The advantage to using this method for gene disruption is in the reusability of the *URA3* selection marker. Approximately 398 bp of the 5'- and 445 bp of the 3'-regions of the *tpi1* gene in *S. cerevisiae* were amplified using two sets of primers, listed in Table 1. The PCR products were introduced into the BamHI/NcoI sites of pWAL200 and pWBR200 to construct pWAL*tpi1*N and pWBR*tpi1*C, respectively. The two separate knockout cassettes were released by digesting pWAL*tpi1*N with BamHI/SalI and pWBR*tpi1*C with SacI/NcoI, and simultaneously transformed into Y2805 (Fig. 2A). URA^+ strains were selected on URA^- medium with 0.1% glucose and 2% ethanol. The insertion of *Kl URA3* on the chromosome was confirmed by the PCR product of the *tpi1* gene analyzed by agarose gel electrophoresis (Fig. 2B). The URA^+ strains were grown in 5-fluoro-orotic acid (5-FOA) medium [4], and then URA^- mutants resulted from eliminating *Kl URA3* were selected (Fig. 2A). The resulting *tpi1* deletion mutation was confirmed (Fig. 2B).

To construct the expression vector for the *mgs* and *gldA* genes, a pESC-URA vector was purchased from Stratagene (San Diego, U.S.A.). Two sets of primers (listed in Table 1) were used for PCR amplification of the *mgs* and *gldA* genes using *E. coli* DH5α chromosome as a template. Each PCR product was cloned into the T-easy vector (Promega, Madison, U.S.A.). The *mgs* gene was inserted into pESC-URA vector using EcoRI/ClaI under a *GAL10* promoter, and the *gldA* gene was inserted under a *GAL1* promoter

Table 1. *Saccharomyces cerevisiae* strains and primer sequences used in this study.

Strains	Genotypes
Y2805	*MATa pep4::HIS3 prb1-d can1 GAL2 his3ura3-52*
Y2805MG	Y2805 with pESC-URA-*mgs & gldA*
CES1	Y2805, but *tpi1*Δ
CES1M	CES1 with pESC-URA-*mgs*
CES1MG	CES1 with pESC-URA-*mgs & gldA*
Primers	**Sequences**
tpiN for BamHI*	5'-GGG ATC CCC CAA ATG GAC TGA TTG-3'
tpiN rev NcoI	5'-GCC CAT GGC GTA ACA AAC CAC CTG TC-3'
tpiC for BamI	5'-GGG ATC CCG TTG ACC AAA TCA AGG-3'
tpiC rev NcoI	5'-GCC ATG GAT CGA CAG CCT TGT G-3'
tpiC rev NcoI*	5'-CGT GAA TGT TCT TGT SSG GGS CGG -3'
gldA for BamHI	5'-GGA TCC ATG GAC CGC ATT ATT CAA-3'
gldA rev KpnI	5'-GGT ACC TTA TTC CCA CTC TTG CAG-3'
mgs for EcoRI	5'-GAA TTC ATG GAA CTG ACG ACT CGC-3'
mgs rev ClaI	5'-ATC GAT TTA CTT CAG ACG GTC CGC-3'

*These primers were used for confirmation of *tpi1* deletion mutation.

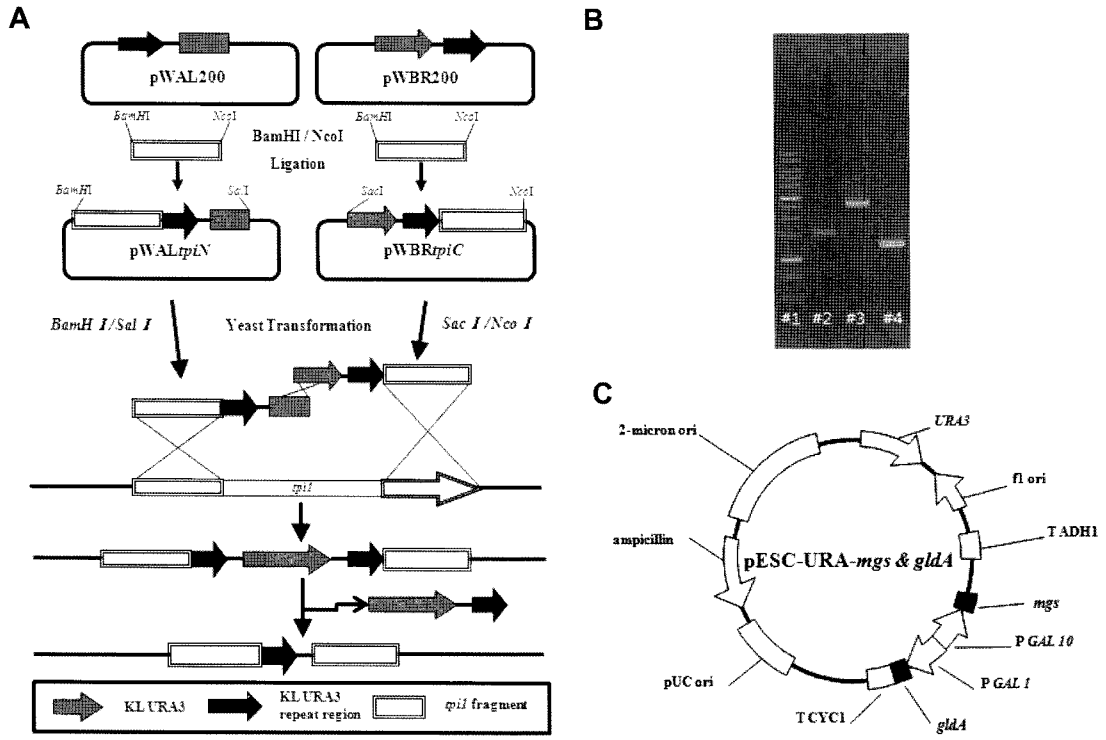

Fig. 2. Gene manipulations used in this study.
A. Scheme of *tpi1* deletion mutation using a homologous recombination method. **B**. Picture of agarose gel electrophoresis of *tpi1* gene regions. Lanes #1, 1 kb ladder; #2, PCR product of *tpi1* gene in Y2805; #3, PCR product in CES1-1 (*tpi1::Kl URA3*); #4, PCR product in CES1 (*tpi1*Δ). **C**. Plasmid map of pESC-URA-*mgs* & *gldA* vector that contains the *mgs* gene under a *GAL10* promoter and the *gldA* gene under a *GAL1* promoter.

using BamHI/KpnI. The resulting vector that includes the *mgs* and *gldA* genes (Fig. 2C) was transformed to *S. cerevisiae* Y2805 or CES1 strain by lithium acetate methods [13].

Media and Cultivation Condition

LB medium (sodium chloride 1%, Tryptone 1%, yeast extract 0.5%, ampicillin 50 μg/ml) was used for *E. coli* selection and YEPD media (yeast extracts 1%, peptone 2%, D-glucose 2%) was used for *S. cerevisiae* Y2805. URA$^-$ medium (Yeast Nitrogen Base without amino acid 0.67%, casamino Acids 0.5%, D-glucose 2%, adenine 0.003%, tryptophan 0.003%) was used for selection and growth of Y2805MG strain. Modified YEPD medium (yeast extracts 1%, peptone 2%, D-glucose 0.1%, ethanol 2%) was used for CES1, and modified URA$^-$ medium with 0.1% D-glucose and 2% ethanol instead of 2% D-glucose was used for selection and growth of CES1M and CES1MG strains. For making solid media, 2% agar was added, and for induction of the *mgs* and *gldA* genes, 1% galactose was added. D-Glucose and agar powder were purchased from Dae-Jung Chemicals Co. Ltd. (Siheung, Korea). Yeast extract, peptone, YNB, tryptone, and CAA were purchased from Becton, Dickinson and company (Franklin Lakes, U.S.A.). Sodium chloride, galactose, adenine, and tryptophan were purchased from Sigma Aldrich (New York, U.S.A.). Flask cultures were performed with 20 ml of media at 30°C, 250 rpm, in 250-ml baffled flasks (PYREX, Seoul, Korea).

Metabolites Assay

Glycerol, methylglyoxal, and 1,2-propanediol were assayed with HPLC ACME-9000 (Young-lin Instrument, Anyang, Korea) using an RI-detector and HPX-87X anion-exchange column (Bio-Rad, Hercules, U.S.A.). Assay was performed at 40°C and 0.01 M sulfuric acid was used as the mobile phase at 0.5 ml/min velocity. Samples for measuring the extracellular metabolite concentration were prepared by taking the medium after centrifugation. Intracellular samples were prepared by boiled ethanol-HEPES buffer cell extraction method [11], followed by filtration with 0.20-μm nylon syringe filters (ALBET, Barcelona, Spain).

RESULTS

Construction of *tpi1* Mutant

To modify the carbon flux to DHAP, deletion of the *tpi1* gene was needed. A homologous recombination method was used to construct the *tpi1* mutant, which used N- and C-terminal fragments of the *Kl URA3* gene in two different vectors, pWAL200 and pWBR200. N- and C-terminal fragments of the *tpi1* gene were PCR amplified and cloned in those vectors (Fig. 2A). Two *tpi1-Kl URA3* fragments were transformed into *URA*$^-$ *S. cerevisiae* simultaneously. The triple homologous recombination allowed the strain to grow in URA$^-$ media. As reported previously, the *tpi1* mutant grows very poorly on glucose minimal medium [9]. Therefore, a URA$^-$ medium with 2% ethanol and 0.1% glucose was used to select the *URA*$^+$ *tpi1* mutant. In the

medium with 2% ethanol and 0.1% glucose, the growth rate of the *tpi1* mutant was recovered, equivalent to that of wild type in 2% glucose medium. The advantage of this deletion method using pWAL200 and pWBR200 is that the *Kl URA3* can be eliminated from the chromosome by another homologous recombination between the repeated regions outside of the N- and C-terminals of the gene, so that the *ura3* gene can be reused as a selection marker. The construction of the *tpi1* deletion mutation was confirmed by PCR amplification with *tpi1* gene-specific primers (Table 1) using the chromosomal DNAs as templates (Fig. 2B).

Glycerol production was expected to increase by the deletion *tpi1* gene owing to the increase of carbon flux to DHAP. When the Y2805 and CES1 strains were grown for 96 h in YEPD and modified YEPD media, respectively, glycerol production in CES1 was much higher than Y2805 in both intracellular and extracellular parts (Fig. 3). The overall glycerol production in strain CES1 was 1.5-fold more than that in Y2805.

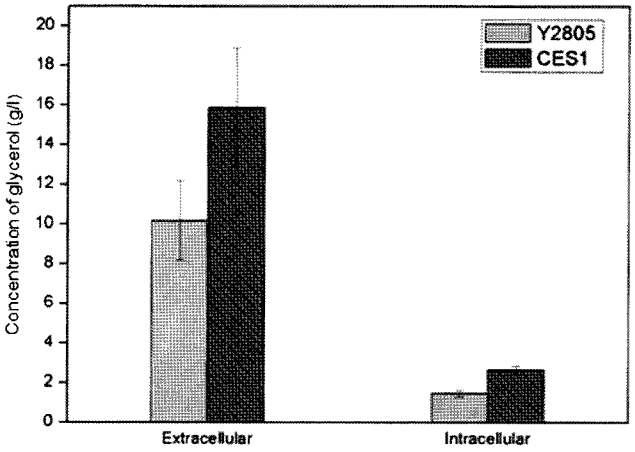

Fig. 3. Glycerol productions of Y2805 (light gray) and CES1 (grey) strains after 96 h flask cultivation.

Redistribution of Carbon Flux

The product of the *mgs* gene synthesizes methylglyoxal from DHAP, and the *gldA* gene forms R-1,2-propanediol from methylglyoxal (Fig. 1). Those genes have been well characterized in *E. coli* [2]. Those genes were PCR amplified from the *E. coli* chromosome and cloned into *S. cerevisiae* to produce 1,2-propanediol using a pESC-URA vector (Fig. 2C). Strains containing the plasmid were selected by URA^- minimal media with 2% ethanol and 0.1% glucose. Since both genes were cloned under *GAL* promoters, 1% galactose was added to the media for *mgs* and *gldA* gene expression.

As the *mgs* gene was cloned and expressed in strain CES1 (CES1M), methylglyoxal was successfully produced in both intracellular and extracellular parts of strain CES1 after 96 h cultivation (Fig. 4). Methylglyoxal was not detected with Y2805, proving that the *mgs* gene product was successfully expressed in CES1M. Glycerol production was significantly decreased in the CES1M strain compared with Y2805. This result suggested that in strain CES1M, the flux was successfully redistributed to methylglyoxal. Interestingly, a small amount of 1,2-propanediol was detected in the intracellular part of strain CES1M, suggesting that there is an endogenous dehydrogenase in *S. cerevisiae* that nonspecifically converts methylglyoxal to 1,2-propanediol.

Next, both the *mgs* and *gldA* genes were cloned in pESC-URA under *GAL1* and *GAL10* promoters and transformed into strain CES1, constructing strain CES1MG. As expected, intracellular and extracellular methylglyoxal concentrations were decreased and a significant amount of 1,2-propanediol was detected (Fig. 4). This result proved the product of the *gldA* gene was also well expressed and converted methylglyoxal to 1,2-propanediol. In strain CES1MG, the

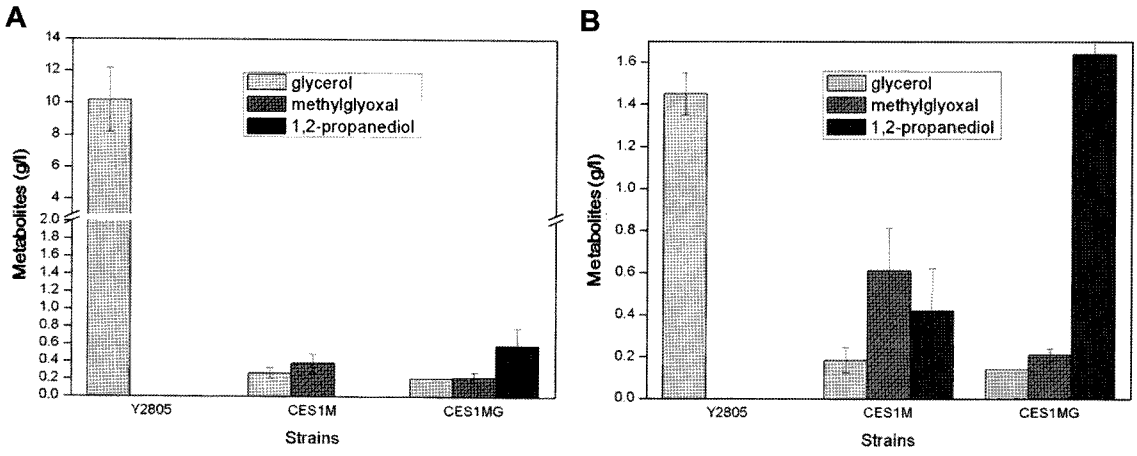

Fig. 4. Extracellular (**A**) and intracellular (**B**) metabolite concentrations of strains Y2805, CES1M, and CES1MG after 96 h flask cultivation. The bars represent the metabolite concentrations of glycerol (light grey), methylglyoxal (grey), and 1,2-propanediol (dark grey).

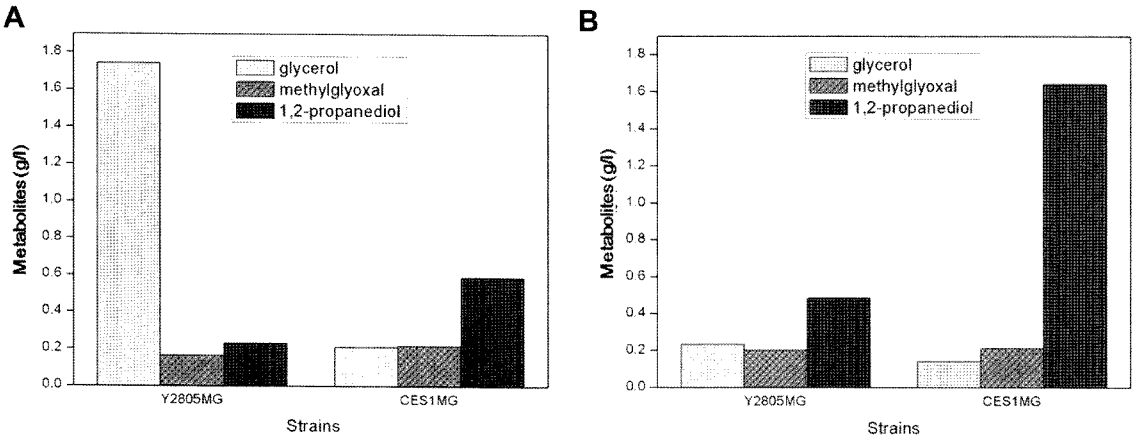

Fig. 5. Extracellular (**A**) and intracellular (**B**) metabolite concentrations of strains Y2805MG and CES1MG after 96 h flask cultivation. The bars represent the metabolite concentrations of glycerol (light grey), methylglyoxal (grey), and 1,2-propanediol (dark grey).

intracellular 1,2-propanediol concentration was about 1.13 times higher than the extracellular one.

Productivity of 1,2-Propanediol in *tpi1* Mutant

Lastly, the 1,2-propanediol productivities of strains Y2805MG and CES1MG were compared. As expected, the CES1MG strain produced a much higher concentration of 1,2-propanediol than Y2805MG after 96 h (Fig. 5). When intracellular and extracellular concentrations were combined, Y2805MG produced 0.38 g/l 1,2-propanediol whereas CES1MG produced 0.89 g/l. The growth rate and extracellular 1,2-propandiol production of Y2805MG and CES1MG were measured for 168 h (Fig. 6). The growth rate showed that *tpi1* mutation made little effect in growth in URA-media with ethanol as a major carbon source. Moreover, the concentration of 1,2-propanediol increased a little bit after 96 h. When intracellular and extracellular concentrations were combined, Y2805MG produced 0.49 g/l 1,2-propanediol, whereas CES1MG produced 1.11 g/l after 168 h cultivation (Fig. 6). It means that our strategy of metabolic engineering successfully redistributed the carbon flux to improve the production yield of 1,2-propanediol.

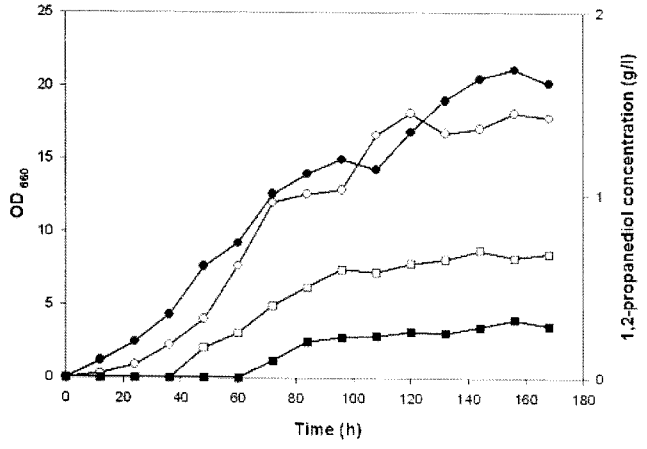

Fig. 6. Growth rate of strains Y2805MG and CES1MG and their 1,2-propanediol production rate during 168 h flask cultivation. The upper part circles represent the growth rate of strains Y2805MG (●) and CES1MG (○). The lower part squares represent the extracellular concentrations of 1,2-propanediol produced from strains Y2805MG (■) and CES1MG (□).

DISCUSSION

Three metabolite concentrations, glycerol, methylglyoxal, and 1,2-propanediol, were measured during the genetic modification for 1,2-propanediol production. Wild-type strain Y2805 produced 8.12 g/l glycerol, but could not produce methylglyoxal and 1,2-propanediol. By deletion of the *tpi1* gene, glycerol production was increased 1.5-fold, proving that *tpi1* deletion shifted the carbon flux to the DHAP side. This increased carbon flux was redirected to methylglyoxal by introduction of the *mgs* gene into the *tpi1* mutant, producing 0.42 g/l methylglyoxal. When both the *mgs* and *gldA* genes were introduced to CES1 strain, 0.24 g/l methylglyoxal and 0.89 g/l 1,2-propanediol were produced. This productivity is much higher than the previous report on the 1,2-propanediol production in engineered *S. cerevisiae* [8], meaning that the *tpi1* mutation contributed quite a lot to the productivity.

A few strategies must be considered for further improvement of 1,2-propanediol productivity. Firstly, the medium must be optimized. The constructed strain was grown in URA^- minimal media containing 2% ethanol, 1% galactose, and 0.1% glucose as carbon sources. However, this medium was designed for selection, not for metabolite production. Careful studies on medium optimization [15] or a fed-batch strategy to supply carbon source [14] is expected to improve the 1,2-propanediol productivity. Next, the efficiency

of the *gldA* gene product should be improved. A metabolic intermediate, such as methylglyoxal, was not converted to the product very efficiently. As pointed out in a previous article [6], molecular evolution of this gene should be considered for further improvement of 1,2-propanediol productivity. Finally, the accumulation of intracellular 1,2-propanediol must be resolved. Intracellular metabolites are much more expensive to separate in bioprocesses. Therefore, an export system of the 1,2-propanediol from the intracellular to extracellular parts must be improved. Fps1P is one of the MIP channels involved in glycerol transport in *S. cerevisiae* [19]. Although the substrate specificity of Fps1p to 1,2-propanediol has never been tested, it is one of the strong candidate transporters for 1,2-propanediol. Therefore, overproduction of this protein might be helpful. Otherwise, the glycerol transporter homologs in other strains, such as *T. thermosaccharolyticum*, should be examined to increase the export rate of 1,2-propanediol.

In this study, *tpi1* deletion mutation and *mgs* and *gldA* overexpression were conducted to increase 1,2-propanediol productivity in *S. cerevisiae*. The carbon flux was successfully increased so that 1.11 g/l of 1,2-propanediol productivity was achieved.

Acknowledgments

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