Korean J. Chem. Eng., 35(8), 1673-1679 (2018)
DOI: 10.1007/s11814-018-0069-x

pISSN: 0256-1115
eISSN: 1975-7220

Engineering of *Saccharomyces cerevisiae* for enhanced production of L-lactic acid by co-expression of acid-stable glycolytic enzymes from *Picrophilus torridus*

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(*Received 13 January 2018 • accepted 30 April 2018*)

Abstract—L-lactic acid, as a monomer of polylactic acid, has attracted much attention because of the growing market for biodegradable bioplastics to reduce landfill waste. As an industrial L-lactic acid producer, *Saccharomyces cerevisiae* is generally used because it survives in low pH. However, in *S. cerevisiae*, production of L-lactic acid causes a decrease in intracellular pH, which leads to slow glycolytic flux, and consequently results in a lower productivity of L-lactic acid. For this reason, yeast strains that maintain their growth and the activities of metabolic enzymes during lactic acid production need to be developed for industrial applications. Herein, acid stable enzymes from acidophilic archaea *Picrophilus torridus* were expressed in L-lactic acid producing *S. cerevisiae* to increase glycolytic flux at low intracellular pH conditions for a higher L-lactic acid titer. Enzymes of lower glycolysis including phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase from *P. torridus* were introduced to develop a novel L-lactic acid producing strain. It was clearly shown that the production of lactic acid in the developed strain increased by 20% compared to the parental strain. To the best of our knowledge, this is the first report of *P. torridus* enzymes used in metabolic engineering to enhance the metabolic flux at a lower intracellular pH. Moreover, it is expected that the new strain will have an enhanced glycolytic flux at a low pH expressing acid stable enzymes that could be used to produce other valuable organic acids with increased titers.

Keywords: L-lactic Acid, *Saccharomyces cerevisiae*, *Picrophilus torridus*, Glycolytic Enzymes, Metabolic Engineering

INTRODUCTION

Lactic acid is a valuable organic acid with extensive applications in cosmetics, pharmaceuticals, and particularly the food industry as an acidulant, flavoring agent, or preservative [1]. Recently, as a monomer of polylactic acid (PLA), lactic acid has received much attention with its sustainability due to the growing market for biodegradable bioplastics [2,3]. Lactic acid bacteria (LAB) such as *lactobacillus* species are the typical hosts for lactic acid production with a large yield through carbohydrate fermentation [4]. Because of the pH sensitivity of LAB, supplementation of large amounts of neutralizing agents such as $CaCO_3$, NaOH, and NH_4OH is necessary for industrial lactic acid production. In this regard, lactic acid production through LAB is limited due to higher production costs caused by the regeneration of precipitate lactate salts [5].

Because of advantages such as growing and surviving in low pH, yeast has been used for lactic acid production, and various yeast species have been metabolically engineered for lactic acid production such as *Kluyveromyces lactis* [6,7], *Torulaspora delbruekii* [7], *Zygosaccharomyces bailii* [8], *Pichia spitipis* [9], *Candida utilis* [10], *Candida boidinii* [11], *Candida sonorensis* [12]. Above all, *Saccharomyces cerevisiae* is the most widely manipulated species for lactic acid production. As the first attempt to construct an L-lactic acid producing strain, heterologous expression of L-lactate dehydrogenase (L-LDH) in *S. cerevisiae* was achieved [13,14]. Several improvements to L-lactic acid production also have been achieved by (i) introducing L-*LDH* genes isolated from various LAB hosts [15], (ii) deleting the pyruvate decarboxylase gene (*PDC1*, *PDC5*, and *PDC6*) or alcohol dehydrogenase genes to reduce ethanol accumulation [16,17], (iii) sorting high acid-tolerant cells which can maintain a higher intracellular pH and produce a large amount of lactic acid [18], (iv) expressing the hexose transporters (HXT1 and HXT7) for glucose uptake [19], (v) expressing the monocarboxylate transporters (JEN1 and ADY2) for export lactic acid [20], (vi) expressing two heterologous lactate dehydrogenase [21], (vii) deleting the S-adenosylmethionine synthetase (*SAM2*) gene involved in phospholipid biosynthesis and membrane remodeling during acid stress [22], and (viii) deleting the NADH-consuming enzymes (NDE1 and NDE2) to enhance the availability of the intracellular redox cofactor for lactic acid production [23].

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Although yeasts have the advantage to grow and produce a large amount of lactic acid at a low pH, the presence of weak organic acids in the medium causes a high level of stress to the yeast cells such as ATP consumption and acidification of the intracellular space, and it was also reported that the intracellular pH (pH_{in}) reached around pH 4 during lactic acid production [24]. At this low pH_{in}, metabolic enzymes could be inactivated [18] and consequently lead to a decrease in the lactic acid production [25]. In this respect, yeast strains that maintain their cell growth and the activities of metabolic enzymes during lactic acid production need to be developed for industrial production without requiring neutralizing agents such as $CaCO_3$. Based on the above premise, yeast cells that maintain a neutral pH_{in} during lactic acid production were isolated, and a higher lactic acid producer (75 g/L) was successfully obtained [18]. The same research group deleted the *SAM2* gene in the phospholipid biosynthesis pathway as a novel target because of its involvement in membrane remodeling during lactic acid stress [22]. However, the production of L-lactic acid was not significantly (1.05-fold) increased in the developed strain, and new trials to increase lactic acid production without neutralizing agents need to be studied for industrial purposes.

In this study, to develop a lactic acid producing strain with a higher yield, we focused on the issue of glycolytic enzymes becoming inactivated in low pH_{in} conditions. To make cells resistant to acidic pH_{in} conditions, we introduced acid-stable enzymes from acid-resistant bacteria into *S. cerevisiae* enhancing the growth and glycolytic flux during lactic acid fermentation and consequently resulting in higher production of lactic acid as well as improved cell growth. As a source of acid-stable enzymes, thermoacidophiles can be considered because they can grow in extremely low pH conditions. Even though they can grow well at very low extracellular pH conditions, the intracellular pHs in most thermoacidophiles are generally maintained at a neutral pH due to their unique pH homeostasis mechanisms [26]. The stability of enzymes originating from those thermoacidophiles is not guaranteed in low intracellular pH conditions, and so, those enzymes cannot be candidates for our purpose. In this respect, thermoacidophilic archaea *Picrophilus torridus* from a dry solfataric field was chosen as the source of the acid-stable enzymes due to its unusual trait: a relatively low intracellular pH (pH_{in}=4.6) compared to other bacteria that have a neutral pH_{in} around 6 [26,27]. As the targeted enzymes, enzymes of lower glycolysis including phosphoglycerate kinase (PGK), phosphoglycerate mutase (GPM), enolase (ENO), and pyruvate kinase (PYK) from *P. torridus* were introduced to develop a novel L-lactic acid producing strain (Fig. 1). Based on this strategy, we carried out

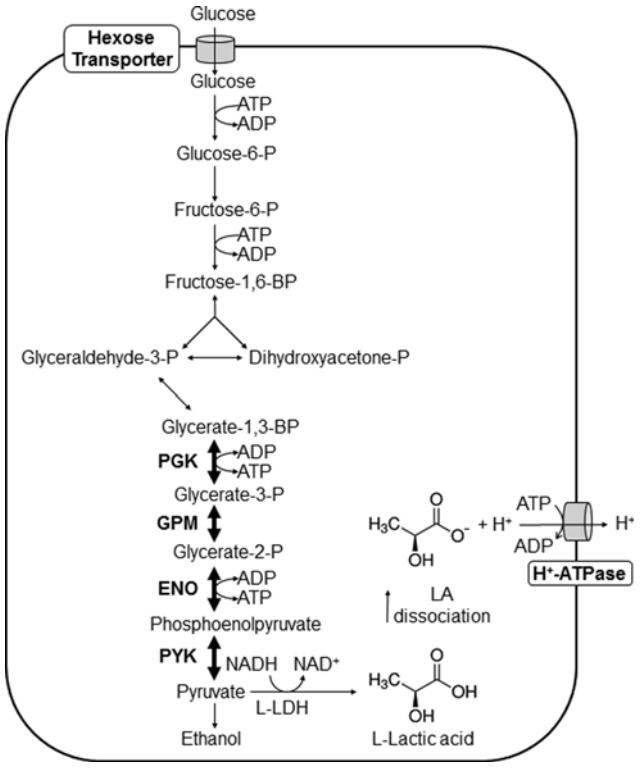

Fig. 1. Biosynthesis pathway of L-lactic acid from glucose in *S. cerevisiae*. The enzymes of lower glycolysis which were co-expressed in the engineered strain are indicated by thick arrows. L-LDH, Lactic acid dehydrogenase.

L-lactic acid fermentation with the developed strain and showed an increase in the productivity as well as in the L-lactic acid yield.

MATERIALS AND METHODS

1. Strains and Media

The bacterial and yeast strains used in this study are listed in Table 1. *E. coli* XL1-blue was used as a host strain for plasmid construction and maintenance. *S. cerevisiae* SP2 strain (CEN. PK2-1D *pdc1Δ:: ldh cyb2Δ::ldh gpd1Δ::ldh*) derived from *S. cerevisiae* CEN.PK2-1D (*MATα ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8^C SUC2*) and expressing the *LDH* gene from *Pelodiscus sinensis* was used as a parental strain for L-lactic acid production [23]. *E. coli* was cultivated in Luria-Bertani (LB) medium composed of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl (BD Difco,

Table 1. *E. coli* and yeast strains used in this study

Strain	Relevant Characteristics	References
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^q Z ΔM15* Tn*10* (Tet^r)]	Stratagene[a]
SP2	CEN. PK2-1D *pdc1Δ:: ldh cyb2Δ::ldh gpd1Δ::ldh*	[23]
SPC	SP2 harboring pRS423-GPD, pRS424-GPD, pRS425-GPD, pRS426-GPD	This study
SPS	SP2 harboring pRS423-PGK(S), pRS424-GPM(S), pRS425-ENO(S), pRS426-PYK(S)	This study
SPP	SP2 harboring pRS423-PGK(P), pRS424-GPM(P), pRS425-ENO(P), pRS426-PYK(P)	This study

[a]Strategene Cloning Systems, La Jolla, CA, USA

Franklin Lakes, NJ, USA) supplemented with 100 mg/L ampicillin (Sigma-Aldrich, St. Louis, Mo, USA). *S. cerevisiae* SP2 was cultivated in YPD medium composed of 10 g/L yeast extract (BD Difco), 20 g/L peptone (BD Difco), and 20 g/L glucose. All SP2-derived strains including SPC, SPS and SPP were cultured in yeast synthetic complete (YSC) medium containing 6.7 g/L yeast nitrogen base without amino acids (BD Difco), 0.6 g/L of CSM-HIS-LEU-TRP-URA (MP Biomedicals, Solon, Ohio, USA), and 20 g/L glucose. YSC media containing 40 g/L or 80 g/L glucose was used for the seed culture or L-lactic acid fermentation, respectively. Histidine and tryptophan (Sigma-Aldrich) were added as supplements with a final concentration of 20 mg/L when required.

2. Plasmid Construction

All plasmids used in this study are described in Table 2. The pRS42X-GPD plasmids with different auxotrophic markers [28,29] were used as the backbone plasmids for the construction of the gene expression plasmids. The genes encoding enzymes of lower glycolysis such as PGK, GPM, ENO, and PYK were amplified from the genomic DNA of *S. cerevisiae* and *P. torridus* by PCR. The genomic DNA of *S. cerevisiae* was extracted from the harvested cells of the SP2 strain with the MasterPure™ Yeast DNA Purification Kit (Epicentre, Chicago, IL, USA). The genomic DNA of *P. torridus* DSM 9790 was purchased from DSMZ (Braunschweig, Lower Saxony, Germany). PrimeSTAR HS Polymerase (Takara Bio Inc., Singa, Japan) was used for the PCR with a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Oligonucleotides used for the amplification of the genes encoding the enzymes of lower glycolysis are listed in Table 3. The PCR fragments were digested with *Bam*HI and *Xho*I restriction enzyme and each of the digested fragments was cloned into the pRS42X-GPD plasmids. For example, the *PGK* genes from *S. cerevisiae* and *P. torridus* were cloned into pRS423-GPD, yielding pRS423-PGK(S) and pRS423-PGK(P),

Table 2. Plasmids used in this study

Plasmids	Relevant characteristics	References
pRS423-GPD	pRS423 containing P_{GPD} and *HIS3* marker	[28,29]
pRS424-GPD	pRS424 containing P_{GPD} and *TRP1* marker	[28,29]
pRS425-GPD	pRS425 containing P_{GPD} and *LEU2* marker	[28,29]
pRS426-GPD	pRS426 containing P_{GPD} and *URA3* marker	[28,29]
pRS423-PGK(S)	pRS423-GPD derivatives, phosphoglycerate kinase 1 gene from *S. cerevisiae*	This study
pRS424-GPM(S)	pRS424-GPD derivatives, phosphoglycerate mutase 1 gene from *S. cerevisiae*	This study
pRS425-ENO(S)	pRS425-GPD derivatives, enolase 2 gene from *S. cerevisiae*	This study
pRS426-PYK(S)	pRS426-GPD derivatives, pyruvate kinase 1 gene from *S. cerevisiae*	This study
pRS423-PGK(P)	pRS423-GPD derivatives, phosphoglycerate kinase gene from *P. torridus*	This study
pRS424-GPM(P)	pRS424-GPD derivatives, phosphoglycerate mutase gene from *P. torridus*	This study
pRS425-ENO(P)	pRS425-GPD derivatives, enolase gene from *P. torridus*	This study
pRS426-PYK(P)	pRS426-GPD derivatives, pyruvate kinase gene from *P. torridus*	This study

Table 3. Oligonucleotides used in this study

Gene	Primer	Sequence (5′→3′)[a]
PGK1 from	F_PGK(S)	TAGT**GGATCC**ATGTCTTTATCTTCAAAGTTGTCTGTCC
S. cerevisiae	R_PGK(S)	ACATGA**CTCGAG**TTATTAGTGATGGTGATGGTGATGTTTCTTTTCGGATAAGAAAGCAACACC
GPM1 from	F_GPM(S)	TAGT**GGATCC**ATGCCAAAGTTAGTTTTAGTTAGACACG
S. cerevisiae	R_GPM(S)	ACATGA**CTCGAG**TTATTACAGATCCTCTTCTGAGATGAGTTTTTGTTCTTTCTTACCTTGGTTGGCAACAG
ENO2 from	F_ENO(S)	ACTAGT**GGATCC**ATGGCTGTCTCTAAAGTTTACGCT
S. cerevisiae	R_ENO(S)	ACATGA**CTCGAG**TTATTATTTATCGTCATCGTCTTTATAATCCAACTTGTCACCGTGGTGGAA
PYK1 from	F_PYK(S)	ATGC**GGATCC**ATGTCGAGATTAGAAAGATTGACCT
S. cerevisiae	R_PYK(S)	GCAT**CTCGAG**TTATTAGTGATGGTGATGATGGTG
PGK from	F_PGK(P)	ACTAGT**GGATCC**ATGAATGGTTCATTCTTTACAATGGACGATTTCG
P. torridus	R_PGK(P)	ACATGA**CTCGAG**TTATTAGTGATGGTGATGGTGATG
GPM from	F_GPM(P)	ACTAGT**GGATCC**ATGAAAAATATTGTATTATTAATCATGGATGGTCTTGGGGATAACC
P. torridus	R_GPM(P)	ACATGA**CTCGAG**TTATTACAGATCCTCTTCTGAGATG
ENO from	F_ENO(P)	ACTAGT**GGATCC**ATGAATTTTGAAATTAAGGATACAAACATAAGAAAG
P. torridus	R_ENO(P)	ACATGA**CTCGAG**TTATTATTTATCGTCATCGTCTTTATAATCTATTAATTCC
PYK from	F_PYK(P)	ACTAGT**GGATCC**ATGTCAAGGACAAAGTTAATAGCAA
P. torridus	R_PYK(P)	GCAT**CTCGAG**TTATTAGTGATGGTGATGATGGTGTGCTGTTATTATTCCTGTATCACCG

[a]Restriction enzyme recognition sites are emphasized in bold

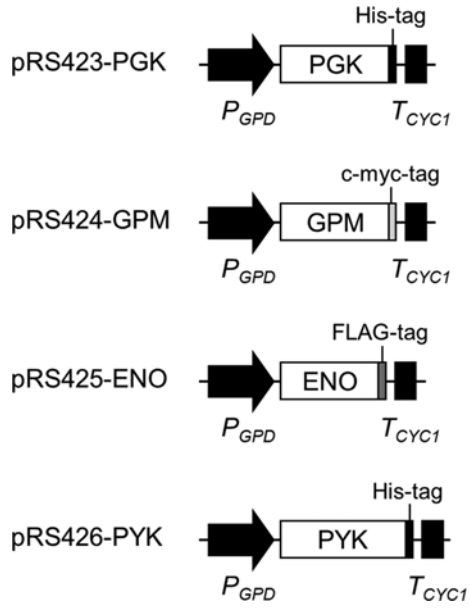

Fig. 2. Schematic diagram of the plasmid constructed in this study. P_{GPD} and T_{CYC1} indicates GPD promoter and CYC1 terminator, respectively. Plasmids for expression of enzymes of the lower glycolysis (PGK, GPM, ENO, and PYK) were illustrated. Each of glycolytic enzymes from *S. cerevisiae* and *P. torridus* is tagged with His-tag (PGK, PYK), c-myc tag (GPM), or FLAG-tag (ENO).

respectively. In a similar way, GPM, ENO and PYK genes were cloned into pRS424-GPD, pRS425-GPD, and pRS426-GPD, yielding pRS424-GPM, pRS425-ENO, and pRS425-PYK, respectively. All plasmids constructed in this study are shown in Fig. 2. Each constructed plasmid was transformed into *E. coli* XL1-blue competent cells with a gene pulser (Bio-Rad, Hercules, CA, USA). After transformation, all constructed plasmids were prepared from *E. coli* XL1-blue cells, and then used for transformation into *S. cerevisiae* SP2 according to the LiAc/PEG/ss-DNA method [30].

3. Flask Fermentation

Cells were cultivated on YSC-HIS-LEU-TRP-URA agar plates composed of 6.7 g/L yeast nitrogen base without amino acids (BD Difco), 0.6 g/L of CSM-HIS-LEU-TRP-URA (MP Biomedicals), 20 g/L glucose, and 20 g/L agar (BD Difco). Cells on plates were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of YSC-HIS-LEU-TRP-URA medium with 40 g/L glucose. After overnight cultivation at 30 °C with shaking (250 rpm), the cells were harvested by centrifugation (3,000 rpm for 10 min. at 25 °C). The cell pellets were resuspended into 250 mL Erlenmeyer flasks containing 50 mL of YSC-HIS-LEU-TRP-URA medium with 80 g/L glucose with an initial optical density (OD) at 660 nm of 0.2. Flask cultivation was done for 120 h at 30 °C with shaking (250 rpm).

4. Analytical Methods

The concentration of residual glucose, L-lactic acid, and ethanol in the extracellular medium was analyzed as described previously [31] with isocratic high-performance liquid chromatography (HPLC) using a Waters 1515 (Waters, Milford, Sweden) equipped with an Aminex HPX-87H ion exchange column 300 mm×7.8 mm (Bio-Rad) and refractive index detector. For the analysis of intracellular metabolites, cells (OD_{660} of 20) were harvested at every time point during cultivation, and cells were extracted using 100% methanol containing 200 μM of D-camphor-10-sulfonic acid as internal standard for signal intensity standardization. The extractant was analyzed using CE-QTOF-MS Agilent 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight mass spectrometer (Agilent Technologies). The system was controlled by Agilent ChemStation software (version B.04.03) for CE and Agilent MassHunter Workstation Data Acquisition software for QTOF-MS (version B.04.00). A fused silica capillary (50 μm i.d.×80 cm length; H3305-2002, HMT, Tsuruoka, Japan) was applied for analytes separation. A commercial anion buffer (H3302-2031, HMT, Tsuruoka, Japan) was used for CE separation as electrolyte. A commercial sheath liquid (H3301-2020, HMT, Tsuruoka, Japan) was delivered at a rate of 1 mL/min. Sample solution was injected at a pressure of 50 mbar for 25 sec. The applied voltage was set at 30 kV. ESI-MS was operated in the negative-ion mode and the capillary and fragmentor voltages were set at 3.5 kV and 101 V, respectively. Nebulizer pressure was configured at 5 psig and heated dry N_2 gas was delivered at a rate of 7 L/min at 300 °C. Exact mass data were acquired at the rate of 2 spectra/sec over a 50-1,000 m/z range.

RESULTS AND DISCUSSION

1. Development of the L-lactic Acid Producing Strain by Co-expressing Acid Stable Enzymes

In the previous study [23], we developed lactic acid producing

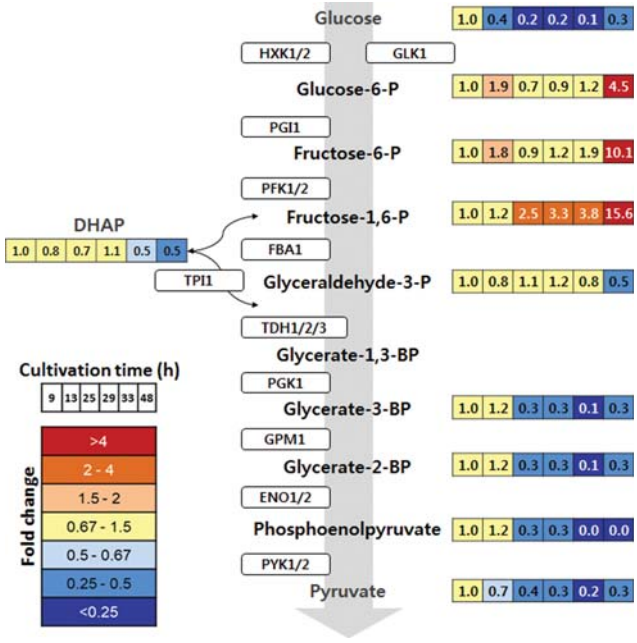

Fig. 3. Analysis of intracellular metabolites. Samples were collected at six different time points (9, 13, 25, 29, 33, and 48 h) and the levels of metabolites in glycolysis pathways were analyzed. Each white box represents enzyme in each step and a gray arrow indicates the main flow of carbon in glycolysis.

strain (SP2) by integrating LDH gene expression system into chromosome. To find the point which is highly sensitive to the pH change, we performed metabolome analysis and found the levels of metabolites (glycerate-3-BP, glycerate-2-BP, phosphoenolpyruvate, and pyruvate) in the lower glycolytic pathways were significantly reduced compared with other metabolites (Fig. 3). Based on those results, we decided to exchange four enzymes in the lower glycolytic pathway with pH-resistant enzymes from thermoacidophilic archaea *P. torridus*. Although *P. torridus* most likely metabolizes carbon sources through a nonphosphorylative Entner-Doudoroff (ED) pathway, all genes required for the Embden-Meyerhof-Parnas (EMP) pathway except for aldolase are present in *P. torridus* [27,32]. From the EMP pathway in *P. torridus*, four enzymes of lower glycolysis, phosphoglycerate kinase (PGK), phosphoglycerate mutase (GPM), enolase (ENO), and pyruvate kinase (PYK), which mediate the conversion of 1,3-bisphosphoglycerate to pyruvate in glycolysis, were chosen to enhance the metabolic flux in glycolysis under lactic acid production in *S. cerevisiae* (Fig. 1). To compare the effect of the enzyme activity at low pH, enzymes of the lower part of glycolysis from *S. cerevisiae* were also cloned into pRS42X-GPD to develop a strain with enhanced glycolytic flux with non-acid stable enzymes. Among the isozymes, the chosen target enzymes from *S. cerevisiae* were phosphoglycerate kinase 1 (PGK1), phosphoglycerate mutase 1 (GPM1), enolase 2 (ENO2), and pyruvate kinase 1 (PYK1). All the enzymes were tagged with His-tag (PGK, PYK from *S. cerevisiae* or *P. torridus*), c-myc tag (GPM from *S. cerevisiae* or *P. torridus*), and FLAG-tag (ENO from *S. cerevisiae* or *P. torridus*). Comparing the enzymes from *S. cerevisiae*, glycolytic enzymes from *P. torridus* have a high similarity in their binding sites except for GPM, which has a difference in the required cofactors (http://www.uniprot.org/uniprot/P00950, http://www.uniprot.org/uniprot/Q6KZJ6). Based on the SP2 strain, three strains were developed: i) an SPC strain which contains all four pRS42X-GPD series and does not produce any recombinant glycolytic enzymes; ii) an SPS strain which contains all four pRS42X-GPD vectors (pRS423-PGK(S), pRS424-GPM(S), pRS425-ENO(S), and pRS426-PYK(S)) for the production of four endogenous glycolytic enzymes of *S. cerevisiae*; iii) an SPP strain which contains all four pRS42X-GPD vectors (pRS423-PGK(P), pRS424-GPM(P), pRS425-ENO(P), and pRS426-PYK(P)) for the production of four glycolytic enzymes of *P. torridus*.

2. Expressing the Enzymes of Lower Glycolysis in the Engineered Strains

Fermentation of lactic acid was carried out in the developed strains, and the protein samples prepared with the 24 h cultured cells grown in YSC medium containing 80 g/L glucose were analyzed with Western Blot following SDS-PAGE. For the detection of the PGK and PYK enzymes, an anti-His monoclonal antibody was used, and the GPM or ENO enzymes were detected with anti-c-myc or anti-FLAG monoclonal antibodies in western blotting. All four enzymes were successfully produced in both SPS and SPP strains; however, we found that the expression levels of the enzymes from *P. torridus* in the SPP strain were much lower than those of the enzymes from *S. cerevisiae* in SPS (Fig. 4). For heterologous gene expression, codon usage is one of the critical points, and we thought that the relatively lower expression levels in the SPP strain might be from unfavorable codons in the cloned genes of *P. torridus* for the *S. cerevisiae* host.

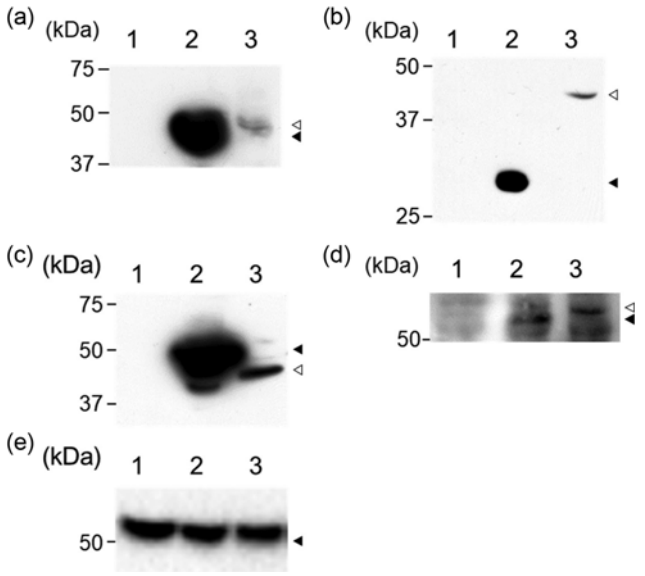

Fig. 4. Western blot analysis of L-lactic acid producing strains expressing enzymes of the lower glycolysis from *S. cerevisiae* and *P. torridus*. Developed strains were cultivated in 250 mL of Erlenmeyer flasks containing 50 mL YSC medium with 8% (w/v) glucose for 24 h. (a) Comparison of expression levels of PGK, (b) GPM, (c) ENO, and (d) PYK from *S. cerevisiae* (closed arrow) and *P. torridus* (open arrow) (e) *S. cerevisiae* alpha-tubulin loading control (closed arrow). Lane 1, SPC; lane 2, SPS; lane 3, SPP.

3. Comparison of L-lactic Acid Production with Strains Expressing the Enzymes of Lower Glycolysis

The SP2 derived strains were cultured in YSC medium with 80 g/L glucose, and the cell density, extracellular metabolites and pH were analyzed periodically. Due to the instability of plasmids, the use of four multi-copy number plasmids may cause strain variability during the cultivation, and to minimize the experimental error due to the strain variability, all experiments were repeated four times with two different transformants. In the early stage (~24 h), the SPP strain producing the glycolytic enzymes of *P. torridus* exhibited a little slower cell growth and glucose consumption than those of the control strain (SPC), but in the late stage, SPP strain grew faster and maximum cell density (OD_{660} of 4.5) was higher than that of SPC (OD_{660} of 4.4) (Fig. 5 and Table 4). SPP strain also exhibited higher cell growth and glucose consumption rate than those of the SPS strain producing the glycolytic enzymes of *S. cerevisiae* (Fig. 5(a) and 5(b)): The maximum cell density of SPS was OD_{660} of 3.6. As one possible reason for slower growth of SPP in the early growth stage (~24 h), we consider the overexpression of four genes in SPP (Fig. 4), which may cause metabolic burden and consequently cell growth and glucose uptake are reduced. However, the expression of acid-tolerant enzymes in SPP makes cells continue to grow in the low pH condition and finally exceed the cell density of SPC in the late growth stage (Table 4). In all cultivations, the extracellular pH began to decrease immediately after inoculation due to the production of lactic acid in the culture media, and after 24 h, the

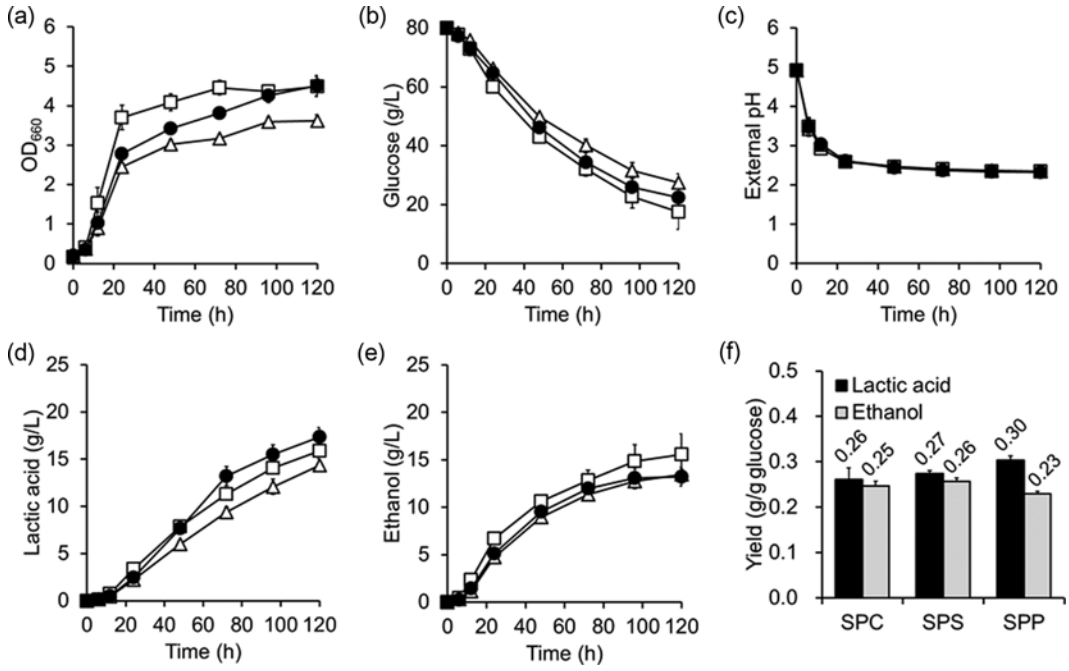

Fig. 5. Comparison of flask fermentations with the L-lactic acid producing strains. SPC (open square), SPS (open triangle), and SPP (filled circle) strains were cultivated in 250 mL of Erlenmeyer flasks containing 50 mL YSC medium with 8% (w/v) glucose for 120 h. Time profiles of (a) cell growth (OD at 660 nm), (b) residual glucose concentration, (c) extracellular pH, (d) L-lactic acid concentration, (e) ethanol concentration in the extracellular medium, and (f) yield of L-lactic acid and ethanol. The data are mean values and standard deviations from four independent experiments.

Table 4. Biomass, L-lactic acid and ethanol productions in developed *S. cerevisiae* strains

Strain	Biomass (OD_{660})	L-lactic acid		Ethanol (g/L)
		Conc. (g/L)	Yield (g/g)	
SPC	4.4±0.25	15.9±0.54	0.26±0.25	15.6±2.16
SPS	3.6±0.16	14.4±0.68	0.27±0.26	13.4±0.40
SPP	4.5±0.26	17.4±0.97	0.30±0.22	13.2±0.97

extracellular pHs were maintained at around pH 2.3 (Fig. 5(c)). From the culture medium, the concentrations of lactic acid were analyzed, and we found that the SPP had a higher production yield of L-lactic acid than that of the other strains (SPC and SPS) (Fig. 5(d)). In the SPP strain, the maximum concentration of L-lactic acid was 17.4±0.97 g/L, which was 1.2-fold and 1.08-fold higher than those of the SPS (14.4±0.68 g/L) and SPC (15.9±0.54 g/L), respectively (Table 4). In the early growth phase (~24 h), the concentration of lactic acid was relatively low (below 5 g/L), and SPC showed better cell growth than SPP (Fig. 5). But, as the lactic acid concentration increased higher than 10 g/L, growth of SPC was significantly reduced while SPP continued to grow and finally higher cell density could be achieved (Table 4). From this time point (48 h), the production of lactic acid in SPP also increased dramatically and higher production could be achieved (Fig. 5(d)). In addition, the SPP strain had a reduced level of ethanol production than that of the SPC strain, and this result could be due to the improved lactic acid production (Fig. 5(e) and Table 4). All strains used in this work contain an L-lactate dehydrogenase (LDH) gene overexpression system for the enhanced production of lactic acid from pyruvate than ethanol. Also, it is known that the LDH is relatively resistant to low pH condition [33]. On the contrary, the alcohol dehydrogenase (ADH), which mediates the biosynthesis of ethanol from pyruvate, is known to be susceptible to low pH condition [34,35]. Under the low pH condition due to high production of lactic acid, active LDH is overexpressed and ADH activity is decreased, so more lactic acid can be synthesized than ethanol. By employing acid-resistant enzymes in SPP, more lactic acid can be synthesized from the improved pool of pyruvate. Glucose yields for L-lactic acid and ethanol were determined for all cultivations, and it was also found that the SPP strain also had a higher glucose yield on L-lactic acid (0.30 g/g) than that of the other two strains (Fig. 5(f) and Table 4). Considering all the data, we conclude that the co-expression of acid-stable enzymes from *P. torridus* in *S. cerevisiae* is effective for improved lactic acid production in the new strain.

CONCLUSIONS

To minimize the adverse effect of low intracellular pH (pH_{in}) on flux in the glycolysis pathway during L-lactic acid production, we introduced the acid-stable enzymes of lower glycolysis from *P. torridus* into *S. cerevisiae*, and successfully demonstrated improved production of L-lactic acid in *S. cerevisiae*. In the present work, the expression levels of all enzymes from *P. torridus* were not enough high in SPP, and we believe that L-lactic acid production can be further increased by optimization of the *P. torridus* gene codons into *S. cerevisiae*-preferable codons. Additionally, a comparable production yield of lactic acid can be achieved through further opti-

mization of the culture conditions in a large-scale bioreactor.

ACKNOWLEDGEMENTS

This work was supported by the Advanced Biomass R&D center (ABC) for Global Frontier Project (Grant no. ABC-2015M3A6A2074238) and by the Samsung Advanced Institute of Technology (SAIT).

NOMECLATURE

ENO : enolase
GPM : phosphoglycerate mutase
PGK : phosphoglycerate kinase
PYK : pyruvate kinase

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