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RESEARCH PAPER

Enhancement of Stress Tolerance and Ethanol Production in *Saccharomyces cerevisiae* by Heterologous Expression of a Trehalose Biosynthetic Gene from *Streptomyces albus*

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Abstract The ability to grow and produce ethanol under stressful conditions is an important factor in industrial bioethanol production. Trehalose is found in many organisms including *Saccharomyces cerevisiae*, and has been known to play an important role in enhancing various types of stress tolerance. In this study, Streptomyces albus trehalose-6-phosphate synthase gene (*salC*) was expressed in *Saccharomyces cerevisiae*, and the recombinant strain with *salC* gene showed significantly improved stress resistances and ethanol production. The stress sensitivity and viability tests indicated that the recombinant had a greater resistance to ethanol than the control. At elevated temperatures, the results of flask cultures showed that the expression of salC played a positive role in protecting cells from heat stress. The recombinant strain was found to consume 100 g/L glucose and to produce 39 g/L ethanol at 40°C with an ethanol yield 6% higher than that of the control strain. In the fed-batch experiment in a bioreactor the recombinant strain produced 69 g/L ethanol with about 16% higher yield and about 13% higher productivity than the control strain. This demonstrated the enhancement of ethanol production capabilities of the recombinant strain under a high-ethanol stress condition.

Keywords: *Saccharomyces cerevisiae*, trehalose, stress resistance, ethanol production

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1. Introduction

It has been traditional to use the yeast *S. cerevisiae* for brewery and fuel ethanol production. Since yeast is usually exposed to stressful industrial conditions such as high temperatures and osmotic pressures, high ethanol concentrations, low nutrients and process interruptions, the ability to grow and produce more ethanol under these stresses is an important trait required for the efficient production of fuel ethanol [1,2].

Trehalose, a non-reducing disaccharide composed of two glucose units, has been found in a wide variety of organisms and known to play a role as a source of energy and carbon. It has been known that trehalose can stabilize proteins, suppress the aggregation of denatured proteins, and protect the cellular membranes [3,4]. Many reports have shown that the genes encoding trehalose biosynthetic enzymes are upregulated under various stresses [5-7]. Moreover, there have been numerous studies on a correlation between stress tolerance and trehalose accumulation in yeast under various stress conditions, such as heat [8-10], freezing [11,12], ethanol [13-15], and acetic acid stresses [16].

The most widely distributed biosynthesis pathway of trehalose is a two-step process. First, trehalose-6-phosphate is formed from UDP-glucose (or GDP-glucose in some

cases) and glucose-6-phosphate. This reaction is catalyzed by trehalose-phosphate synthase (TPS, encoded by *tps1* in yeast). Then, this compound is converted to trehalose by trehalose-phosphate phosphatase (TPP, encoded by *tps2* in yeast) [17].

In a previous study performed by our research group, five genes that included *salB, salC, salD, salE,* and *salF* from *Streptomyces albus* (ATCC 21838) were isolated and the five-gene module revealed functional resemblance with trehalose biosynthetic enzymes through sequence analysis. It was demonstrated that the *Streptomyces lividans* TK24 transformants contain in this module produced noticeable amount of trehalose and showed less cell lysis at the later phase of cultivation than the wild type [18].

In the preliminary study, it was investigated as to whether heterologous expression of *salC* and/or *salE* genes, which were expected to encode the key enzymes in trehalose synthesis, could enhance stress tolerance in *S. cerevisiae*. We constructed four expression vector systems with different promoter-product gene combinations: GAL10-*salC*, GAL10-*salE*, GAL10-*salC*/*salE* and GAPDH- *salC*. Only the transformant containing GAL10-*salC* showed a significantly increased cell growth, trehalose accumulation, and ethanol and heat tolerance.

In this study, we have characterized the *salC* gene, the putative trehalose-phosphate synthase and in depth evaluated effects of its expression on heat and ethanol tolerance, and eventually ethanol production in *S. cerevisiae*. This report describes the first application of a newly characterized trehalose biosynthetic enzyme from *Streptomyces* towards improving a yeast strain.

2. Materials and Methods

2.1. Strains, plasmids, and culture media

All the strains and plasmids used in this study are listed in Table 1. *Streptomyces* strain was maintained on R2YE agar medium and cultivated in R2YE liquid broth at 28ºC [19]. *E. coli* was maintained and cultivated at 37ºC in Luria-Bertani (LB) medium [20]. If necessary, 50 mg/L ampicillin was added to the LB medium. For strain construction of *S. cerevisiae*, YPAD medium (2% glucose, 2% bacto peptone, 1% bacto yeast extract, and 0.02% adenine) and SC-Ura medium (0.67% yeast nitrogen base without amino acids, 0.192% yeast synthetic drop-out medium supplements without uracil and 2% glucose) were used. The yeast strains were maintained on SC-Ura agar plate. For seed culture, SC-Ura medium and YPD medium (2% glucose, 2% bacto peptone, and 1% bacto yeast extract) were used. YNBPD medium (2% glucose, 0.67% yeast nitrogen base without amino acids, and 2% bacto peptone) was used for flask cultures to test for stress. A concentrated medium called 'fermentation medium' (10% glucose, 2% peptone, 0.6% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, and 0.12% $MgSO_4{\cdot}7H_2O$) was used in flask cultures for ethanol production. A modified fermentation medium and a feeding solution containing 50% glucose were used for feed-batch culture. The modified fermentation medium contains 0.75% yeast extract, 0.75% peptone, 0.6% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.5% $MgSO_4{\cdot}7H_2O$, and 5% glucose (pH 5.5).

2.2. Enzymes and chemicals

Restriction endonucleases and other DNA modifying

Table 1. Strains and plasmids used in this study

Strains or plasmids	Properties or product	Reference or source
Strains		
*S. albus*ATCC 21838	Wild-type	[37]
E. coli DH5α	F^-, $_{\varphi}$80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Stratagene
S. cerevisiae 2805 Δ*gal80*	*MATα pep4::HIS3 prb- Δ1.6R can1 his3-20 ura3-52,* Δ*gal80*	provided by Dr. E.-S. Choi (KRIBB) [23]
Plasmid		
pGEM-T	TA cloning vector, *LacZ*	Promega
pET28a	T7 promoter, expression of His-fagged fusion protein	Novagen
pSC1	pGEM-T vector carrying a *salC* gene from *S. albus* ATCC 21838	This study
pSC2	pET28a(+) carrying a *salC* gene, a 1.47 kb NdeI-EcoRI fragment, from pSC1	This study
YEGα-HIR525	2 μ-based high-copy-number plasmid, GAL10 promoter, GAL7 terminator, URA3 selection marker, MFα pre-pro secretion signal (ppL), hirudin expression	provided by H. Kang (KRIBB)
YEG	ppL and hirudin gene deleted YEGα-HIR525, empty vector	This study
YEG-SC	YEG carrying *salC* gene	This study

enzymes were purchased from Takara Shuzo Inc. (Tokyo, Japan). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primers for PCR-based cloning were purchased from DyneBio Inc. (Seoul, Korea) or Genotech Co. (Daejeon, Korea).

2.3. Heterologous expression and purification of recombinant His_6-tagged *salC* in *E. coli*

The *salC* gene was amplified by PCR using the genomic DNA of *Streptomyces albus* as template and primers, 5'-GATGGAGAGGGCATATGGCACGTCAG-3' containing a NdeI site and 5'-GCCGAATTCCCTCACGCGTCATGC GCAC-3' containing a EcoRI site. The PCR product was cloned into the pGEM T easy vector (Promega), resulting in pSC1. The NdeI-EcoRI fragment carrying the *salC* gene from pSC1 was then ligated into pET28a expression vector (Novagen). pSC2 was transferred into *E. coli* BL21(DE3)pLysS by electroporation. The transformant was cultured in 100 mL of LB medium, supplemented with kanamycin (50 g/mL) and chloramphenicol (25 g/mL), at 37°C and 200 rpm, to an OD_{600} of 0.5. Then, IPTG (0.2 mM) was added, and the culture was allowed to grow for an additional 24 h at 16°C. Cells were harvested by centrifugation (5,000 × *g*, 10 min) and disrupted by French press in disruption buffer (20 mM Tris-Cl, pH 7.9, containing 500 mM NaCl, 5 mM imidazole). The cell debris was removed by centrifugation and the protein solution was mixed with Ni^{2+}-NTA agarose. The Ni^{2+}-NTA agarose was washed three times with the same buffer and the His_6-tagged protein was eluted with buffer containing imidazole (250 mM). The eluted protein was dialyzed in a dialysis buffer (25 mM Tris-Cl, pH 7.8, containing 10 mM $MgCl_2$). Protein concentration was measured using a Bradford protein microassay kit (Bio-Rad) with bovine serum albumin as the standard protein.

2.4. Enzyme assay for trehalose-6-phosphate synthase

The reaction mixture for enzyme assay consisted of 25 mM Tris-Cl buffer (pH 7.8), 10 mM $MgCl_2$, 5 mM dNDP-glucose, and 8 mM glucose-6-phosphate as the substrate. One hundred μL of reaction mixtures containing 25 μL of enzyme solution (2 mg/mL) were incubated for 1 h at 30°C. Two μL of Alkaline phosphatase (calf intestine, 30 U/μL, Takarashozo Inc., Tokyo, Japan) was added to the reaction mixture and incubated for 1 h at 30°C After ultrafiltration (3kDa cut-off, Millipore, USA), 2 μL each of reaction mixture was spotted onto thin-layer chromatography (TLC) plates (Silica 60; Merck). As the standard, 2 μL of 10 mM trehalose was spotted. The TLC plates were developed in 1-butanol-ethanol-water (5:3:2), air dried, and sprayed with staining solution (5% hydrogen sulfate, 5% acetic acid, 0.5% anisaldehyde in deionized water). The sugar spots were made visible by charring (120°C, 3 min).

2.5. Construction of *salC* overexpression strains of *S. cerevisiae*

YEGα-HIR525 was used as the expression vector. The control vector, YEG, was obtained by *Eco*RI and *Sal*I digestion of YEGα-HIR525, end-filling the sites with Klenow fragment and blunt-end ligation of end-filled sites of vector. The *salC* DNA fragment amplified by PCR using the forward primer (5'-GAA TTC ATG GCA CGT CAG AGT-3') and reverse primer (5'-GGT TAC CCT ACT ATC ACG CGT CAT GCG C-3') was cloned into the *Eco*RI and *Bst*EII sites of a YEGα-HIR525, downstream to GAL10 promoter and the resulting plasmid was named YEG-SC. The Δ*gal80* mutant of *S. cerevisiae* cells were transformed by the lithium acetate method described by Gietz, Schiestl *et al.* [21] with YEG-SC or YEG (control) vector and transformant cells were isolated by plating on SC-Ura agar plates at 30°C for 3 day.

2.6. RNA isolation and RT-PCR

YEG or YEG-SC vector containing yeast strain was grown in 50 mL YNBP medium with 2% glucose at 30°C for 12 h. The initial cell density was adjusted to an OD_{600} of around 0.4. Cells were harvested at 6 h and at 12 h after inoculation, and resuspended in RNAprotect™ Bacteria Reagent (Quiagen, Netherlands) for 5 min. After centrifugation, the cell pellet was stored at -70°C. RNeasy Mini kit (Quiagen) was used for RNA isolation according to the manufacturer's instructions. Contaminant DNA in the sample was eliminated by using RNase-free DNase (Quiagen). The total RNA was quantified using a NanoDrop ND-1000 (Nanodrop, USA). The purity of the RNA preparation was checked by measuring the absorbance ratio at 260/280 nm.

For RT-PCR analysis, 300 ng of the RNA sample was reverse-transcribed and the reverse-transcribed cDNA was used as a template for amplification by PCR using the ONE-STEP RT-PCR PreMix kit™ (Intron Biotechnology, Korea) according to the manufacturer's instructions. Primers for *salC* were 5'-GAG GTG CTG TGG CAC ACC AT-3' and 5'-CGC GGA AGG CCC TTC AGC GC-3'. Primers for the expression of *ura3-1* used as the internal standard for RNA analysis were 5'-CGT GCT GCT ACT CAT CCT AGT CC-3' and 5'-CTG TTG ACC CAA TGC GTC TCC C-3'. Reaction conditions were as follows: reverse transcription reaction at 45°C for 30 min; initial denaturation at 94°C for 5 min; 25 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec; and a final extension step of 5 min at 72°C.

2.7. Stress sensitivity tests on plates

For comparing the growth phenotypes of the recombinant

strains with the control strain under various stress conditions, cells of each strain were precultured in SC-Ura medium at 30°C for 12 h. Approximately 10^7, 10^6, 10^5, or 10^4 cells were spotted on SC-Ura agar plate. Cells were grown at 30°C on solid medium supplemented with 0 ~ 15% (v/v) ethanol or 0 ~ 20 mM furfural for 3 ~ 5 days. For heat stress test, cells were grown at 37°C for 3 days or 50°C for 2 h.

2.8. Culture conditions

2.8.1. Flask culture stress test

Seed culture was incubated in SC-Ura medium at 200 rpm and 30°C for 16 h. The seed culture was transferred into 100 mL of YNBPD medium at OD_{600} of 0.5 and was incubated at 160 rpm and 30°C. For evaluating the growth behavior under ethanol stress, we added pure ethanol to the 4 hour-grown culture (early-exponential phase) up to an ethanol concentration of 5 or 10%. For evaluating the growth behavior and ethanol production at an elevated temperature, the 4 hour-grown culture (early-exponential phase) was transferred to other shaking incubators maintained at 40 and 42°C, respectively. Experiments were performed in triplicate.

2.8.2. Viability of cells under stress conditions

The seed culture was incubated in SC-Ura medium at 200 rpm and 30°C with shaking. At 16 h of the incubation, the seed culture was inoculated in 50 mL of YNBPD medium to achieve an OD_{600} of around 0.5 and was grown for 4 h at 160 rpm and 30°C with shaking. Cells were harvested, resuspended in Phosphate- buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4; pH 7.4) with 2% glucose, and then the cells were incubated for 12 h. For ethanol stress test, we added 100% ethanol to the PBS to concentrations of 0, 6, 8, 10, and 12% (v/v). The cells were incubated at 30, 35, 40, 45, and 50°C for heat stress test. Following these treatments, viability was determined by the alamarBlue (TREK Diagnostic Systems, OH, USA) staining. The treated cells were harvested and 1 mL of PBS containing 10% (v/v) alamarBlue was added. The mixture was incubated for 4 h before taking the spectrophotometric readings. Survival ratio was determined as the relative values to the non-stressed cells. Experiments were performed in triplicate.

2.8.3. Flask culture for ethanol production test

Batch culture was performed in a 500 mL Erlenmeyer flask. Cells grown in SC-Ura medium were used to inoculate YPD medium for the seed culture. The seed culture was incubated at 200 rpm and 30°C with shaking. At 16 h of the incubation, the seed culture was inoculated in 300 mL of the fermentation medium to achieve an OD_{600} of around 1.0 and was grown at 160 rpm and 30 or 40°C with shaking. Experiments were performed in triplicate.

2.8.4. Fed-batch culture in bioreactor

To investigate the performance of the recombinant yeast at an elevated level of ethanol concentration, it was necessary to accumulate ethanol to a high level. For this purpose, fed-batch ethanol fermentation was conducted in a 5.0 L jar fermentor (BioCNS, Korea) with a working volume of 2.0 L. The temperature, agitation speed, and aeration were controlled at 30°C, 200 rpm, and 1 mL/min, respectively. In the beginning a batch culture was conducted with the modified fermentation medium. At the end of the batch period, intermittent feeding of a concentrated glucose solution (500 g/L) was started to maintain the glucose concentration in the range of 10 ~ 50 g/L. Industrial-grade glucose and nitrogen sources were used in the fed-batch fermentation.

2.9. Analytical methods

2.9.1. Cell growth and metabolites in culture broth

Yeast cell growth was monitored by measuring OD_{600} and dried cell weight (DCW). In measuring DCW, samples were washed and the wet cell pellets were transferred to preweighed plastic dishes, placed in the 80°C drying oven for 24 h, and cooled at room temperature. DCW was determined by the difference in weight of the dishes only and the dishes with cells. Ethanol concentration was determined by using YSI 2700 Select Biochemistry Analyzer (YSI, Ohio, USA). Glucose, galactose, and extracellular trehalose concentration was analyzed by high-performance liquid chromatography (Waters, Milford, USA) using Aminex HPX-87P column (Bio-Rad, Hercules, USA). The column was eluted with water as mobile phase and a flow rate of 0.6 mL/min at 85°C.

2.10. Measurement of intracellular trehalose content

Harvested cells were washed and suspended in 0.5 mL of distilled water, and then incubated for 15 min in a boiling water bath to extract intracellular small molecules, including trehalose. After centrifugation (10 min at 8,000 rpm), the supernatant was analyzed. Intracellular trehalose analysis was performed with a HPLC system consisting of a DIONEX Ultimate 3000 chromatograph equipped with a Electrochemical Detector ED50, a CarboPac™ PA1 column (4 mm × 270 mm), and a CarboPac™ PA1 guard column (4 mm × 500 mm). The column was eluted with 50 mM NaOH solution, as mobile phase at a flow rate of 1 mL/min at 30°C.

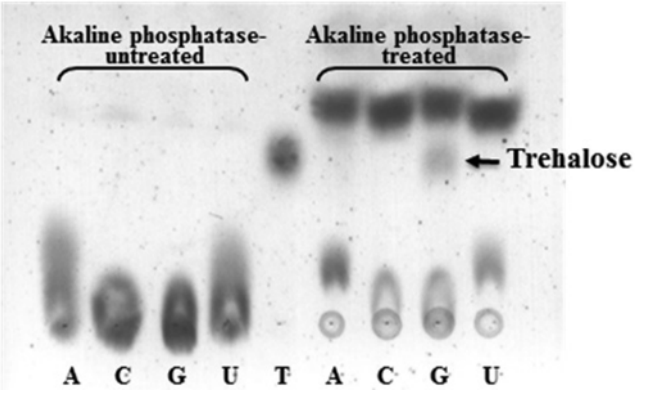

Fig. 1. TLC analysis of the products after trehalose-6-phosphate synthase reaction with SalC (left) and then further reacted with calf intestine alkaline phosphatase (right).T, trehalose (standard); A, ADP-glucose; C, CDP-glucose; G, GDP-glucose; U, UDP-glucose as the substrate.

3. Results and Discussion

3.1. Characterization of the *salC* protein as a trehalose-6-phosphate synthase

It was expected that *salC* gene would encode trehalose-6-phosphate synthase (TPS) one of the enzymes involved in trehalose biosynthetic pathway, as mentioned earlier according to homology search results [18]. For the functional elucidation of the encoded protein, His_6-tagged SalC protein was expressed in a soluble form in *E. coli* by the transformation of the expression plasmid pSC2. Purification of His_6-tagged protein by Ni^{2+}-NTA yielded a single band in SDS-PAGE. The purified SalC was used for TPS assay using glucose-6-phosphate as the substrate and dNDP-glucose as the glucose donor. The thin layer chromatography (TLC) analysis was failed to detect the product (trehalose-6-phosphate) probably due to the high polarity of the phosphate group (Fig. 1, left). Therefore, another step of dephosphorylation of trehalose-6-phosphate was done by the addition of alkaline phosphatase (calf intestine, Takarashuzo Inc., Tokyo, Japan). The dephosphorylated product, trehalose, could be successfully detected by TLC under the same condition only when GDP-glucose was used as the glucose donor (Fig. 1, right). This result clearly indicated that SalC was a TPS that has aspecificity to GDP-glucose.

3.2. Heterologous expression of *salC* in *S. cerevisiae* and its effects on growth properties and intracellular trehalose accumulation

In the preliminary study, *salC* and *salE* genes from *Streptomyces albus*, which were expected to encode the key enzymes in trehalose synthesis, were expressed in a heterologous manner, either individually or together in *Saccharomyces cerevisiae* YPH500 strain. Different vectors

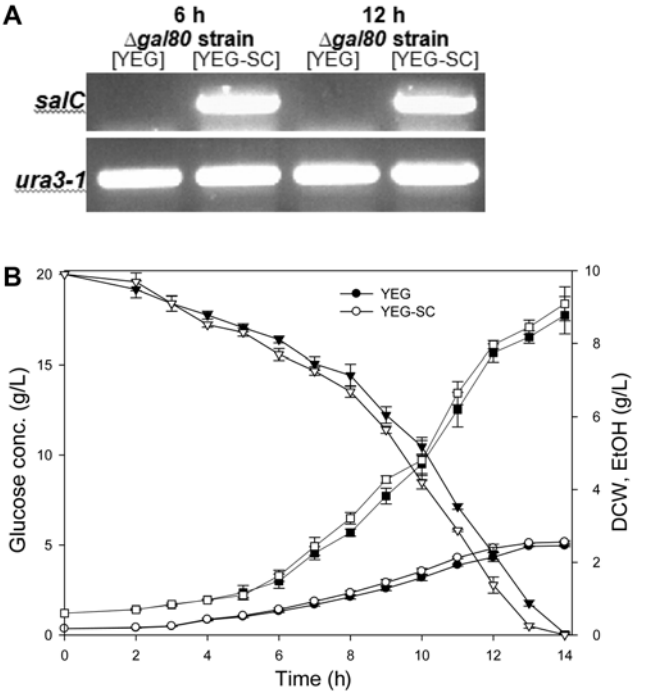

Fig. 2. (**A**) RT-PCR analysis of *salC* gene expression in Δ*gal80* mutant of *S. cerevisiae*. (**B**) Growth (circle), glucose concentration (triangle), and ethanol production (square) of YEG-SC (open) and the control (filled) strain grown in YNBPD medium.

for heterologous expression of trehalose biosynthetic genes in *S. cerevisiae* were constructed, and preliminary performance tests were carried out. In most cases, the expression of trehalose gene(s) under the control of GAL10 promoter increased cell growth, trehalose content and heat and ethanol tolerance. The strain containing the *salC* gene only was shown to be the most efficient to enhance cell growth rate, trehalose accumulation, and heat/ethanol tolerance compare to other transformants. Expression of both *salC* and *salE* genes appeared to be a burden on the cell. Therefore, a transformant containing the expression vector with GAL10 promoter and *salC* gene (YEG-SC) was selected for subsequent study. However, GAL10 promoter required galactose and is tightly repressed by glucose [22], while the galactose utilization and fermentation efficiency in *S. cerevisiae* is normally low and galactose is much more expensive than glucose, which is prohibitive for bioethanol production. Thus, we used a Δ*gal80* mutant of *S. cerevisiae* 2805 as the host, which was reported not to require galactose as an inducer [23].

The Δ*gal80* mutant of *S. cerevisiae* transformed with YEG (hereafter, *YEG* or *the control strain*) or YEG-SC (hereafter, *YEG-SC* or *the recombinant strain*) was grown in YNBPD medium. The expression of *salC* gene in YEG-SC strain was confirmed by RT-PCR analysis (Fig. 2A). Cell growth behavior and ethanol production of the transformants

Table 2. Intracellular trehalose content of YEG-SC and the control strain

	YEG	YEG-SC
6 h	0.73 ± 0.02	1.88 ± 0.02
12 h	0.48 ± 0.01	0.59 ± 0.01

were also examined (Fig. 2B). Ethanol production by YEG-SC strain evaluated at 14 h (9.1 g/L) was 5% higher than that of the control strain (8.8 g/L). It was also observed that the intracellular trehalose content at 6 and 12 h was considerably higher in YEG-SC than the control (Table 2). The *salC* gene was found to encode a GDP-glucose utilizing TPS. The host, *S. cerevisiae*, however is known to have a TPS system specific to UDP-glucose only [17]. It was speculated that the expression of *salC* augmented the trehalose biosynthetic pathway in the host strain resulting in increased amount of intracellular trehalose.

3.3. Enhanced stress tolerance of the transformant

It is known that accumulated trehalose in cells is an important factor for resistance of yeast strains to various stress conditions [1]. We examined the effects of *salC* gene expression in *S. cerevisiae* to improve stress tolerance by means of several stress sensitivity tests.

3.3.1. Growth in plate cultures under various stress conditions

Effects of *salC* gene expression on yeast cell growth patterns in plate cultures under various stress conditions

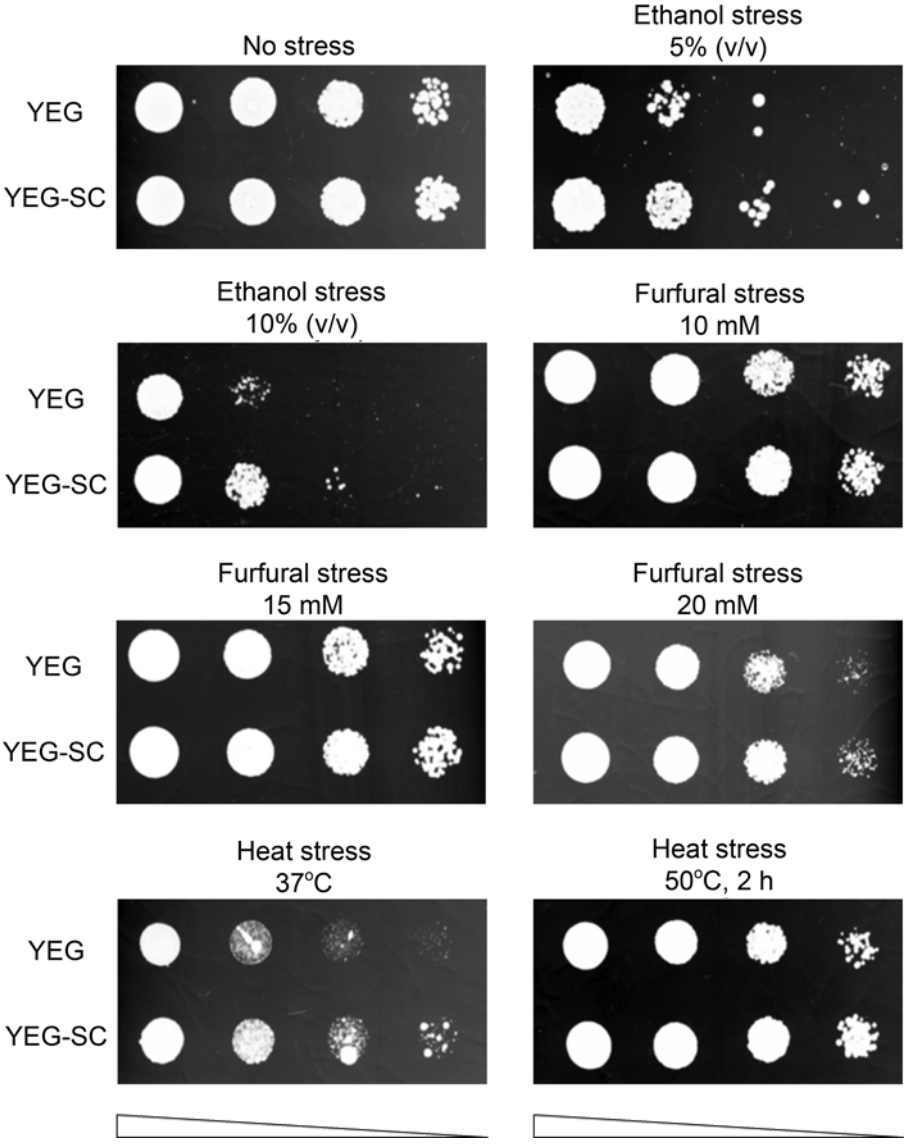

Fig. 3. Comparison of growth patterns of YEG-SC and the control strain under various stress conditions.

were visually compared. A culture grown in the SC-Ura liquid medium was diluted and dropped on a SC-Ura agar plate. The control and recombinant strains were grown under ethanol, heat or furfural stresses. As shown in Fig. 3, both of the strains grew normally under the no-stress condition. YEG-SC strain showed a quite higher level of survival on an agar plate supplemented with up to 10% (v/v) ethanol than the control. The growth patterns of both strains were not quite different from each other under 10 and 15 mM furfural stresses, respectively. In the case of 20 mM furfural stress conditions, however, YEG-SC strain showed a slightly better growth. YEG-SC strain grew much better at 37°C than the control, although cells of both strains grew much more slowly at 37°C than under the no-stress condition of at 30°C. In the case of exposure to a lethal level of temperature than is 50°C for 2 h, YEG-SC strain showed a slightly better growth.

3.3.2. Effects of ethanol concentration and temperature on viability

Cell viability was measured to evaluate the stress tolerance to ethanol concentration and temperature. Fig. 4A shows the relative viability of both strains after being exposed to 0, 6, 8, 10, and 12% (v/v) ethanol for 12 h. The cell viability of the control strain significantly decreased in the presence of over 10% ethanol, while that of YEG-SC strain was maintained to be about 94% even under 12% ethanol stress.

The relative viability of the strains after incubation at 30, 35, 40, 45, and 50°C for 12 h is given in Fig. 4B. Although the cell viability of both strains declined with increasing temperature, YEG-SC strain showed viability a slightly higher than the control: about 5% higher at 45°C, as an example.

In the viability test with an ethanol or heat stress also, the expression of *salC* showed the most significant improvement for ethanol tolerance, the most critical process parameter in ethanol fermentation. The relative viability of YEG-SC strain was approximately 1.8-fold greater than that of the control in the presence of 12% ethanol. Ethanol is the main source of stress during fermentation that affects the rigidity of cytoplasmic membranes, endocytosis and synthesis of stress proteins [1,24]. In the zymolyase sensitivity test for monitoring cell wall integrity known to be needed for ethanol tolerance in yeast [25], the recombinant strain showed a better resistance to zymolyase (data not shown).

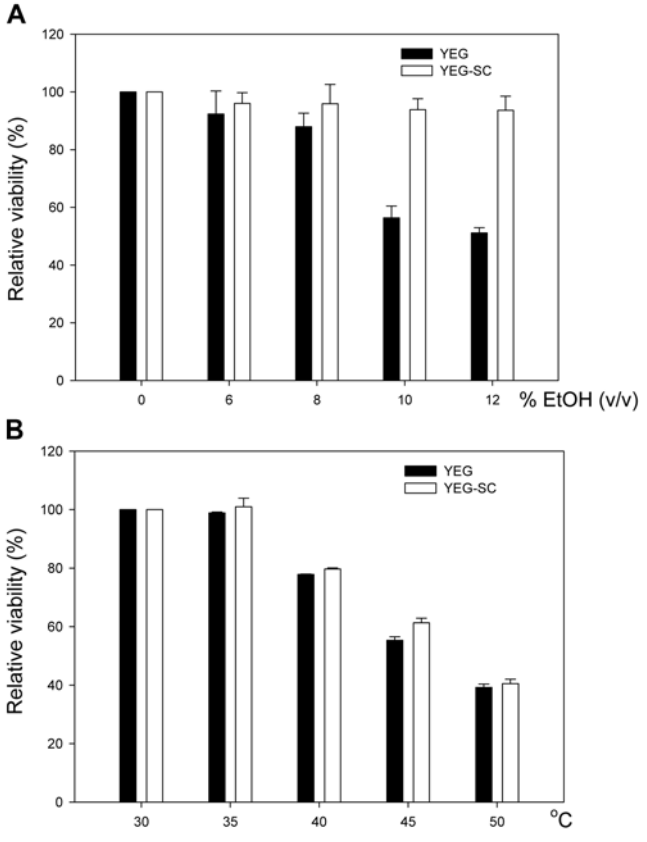

Fig. 4. Cell viability of the *salC* expression (open bar) and the control (filled bar) strain under (**A**) ethanol stress and (**B**) heat stress, respectively.

3.4. Comparison of growth and ethanol production behavior under ethanol or heat stress conditions under low glucose concentration

To compare the growth behavior of the yeast strains in liquid culture under ethanol stress, we monitored the cell concentration of the recombinant and control strains in the presence of 5 and 10% (v/v) ethanol. In all cases, YEG-SC strain showed a slightly higher cell growth than the control (Table 3). The final cell concentration of YEG-SC strain was 6% higher than that of the control with 5% ethanol. In the presence of 10% ethanol, the growth of both strains was severely suppressed. Although *salC* expression was found to have a profound effect on ethanol resistance in the stress sensitivity and viability test, it was not effective in preventing cell growth retardation under ethanol stress in flask cultures. In addition, the effects of heat stress were examined. As shown in Table 3, cell growth of YEG-SC strain was higher than those of the control under heat stress conditions at 40 and 42°C. Similarly to the results with ethanol stress, the cell growth of both strains was significantly inhibited by heat stress. However, the cell concentration of YEG-SC strain was slightly higher than that of the control. It was observed that ethanol production was less affected by heat stress than cell growth. The amount of the ethanol produced by YEG-SC strain at 40 and 42°C were about 5 and 4% higher than by the control, when it reached the highest concentration.

Table 3. Key physiological data evaluated in the comparison of YEG-SC and the control strain under ethanol or heat stress conditions under low glucose concentration

Condition	EtOH added (% v/v)	Temp. (°C)	Strain	Max. cell conc. (g/L)	Max. EtOH conc. (g/L)	EtOH yield (g EtOH/g Glc)
No stress	0	30	YEG	2.46 ± 0.03 (14 h)	8.77 ± 0.51 (14 h)	0.41
			YEG-SC	2.56 ± 0.03 (14 h)	9.09 ± 0.47 (14 h)	0.43
EtOH stress	5	30	YEG	1.72 ± 0.07 (24 h)	-	-
			YEG-SC	1.82 ± 0.04 (24 h)	-	-
	10	30	YEG	0.40 ± 0.01 (18 h)	-	-
			YEG-SC	0.44 ± 0.01 (18 h)	-	-
Heat stress	0	40	YEG	1.55 ± 0.06 (14 h)	8.01 ± 0.43 (14 h)	0.38
			YEG-SC	1.60 ± 0.09 (14 h)	8.40 ± 0.53 (14 h)	0.40
	0	42	YEG	0.86 ± 0.09 (16 h)	6.01 ± 0.59 (24 h)	0.25
			YEG-SC	0.91 ± 0.09 (14 h)	6.26 ± 0.90 (24 h)	0.27

Table 4. Key physiological data evaluated in the flask cultures under high glucose concentration at 30 and 40°C

Temp. (°C)	Strain	Max. cell conc. (g/L)	Max. EtOH conc. (g/L)	EtOH yield (g EtOH/g Glc)
30	YEG	6.46 ± 0.25 (24 h)	40.70 ± 0.51 (24 h)	0.40
	YEG-SC	6.49 ± 0.31 (24 h)	40.73 ± 0.31 (24 h)	0.40
40	YEG	2.95 ± 0.11 (30 h)	36.90 ± 1.27 (30 h)	0.39
	YEG-SC	3.10 ± 0.14 (30 h)	39.11 ± 0.53 (30 h)	0.44

3.5. Flask cultures under high glucose concentration

To assess the ethanol production performance of the recombinant strain under an elevated level of glucose concentration, which would be closer to the condition in the field than the earlier case of 20 g/L of glucose, the fermentation medium containing 100 g/L of glucose was used in batch culture. We compared cell growth, glucose consumption, and ethanol production levels of the recombinant and control strains at 30 and 40°C, respectively (Table 4). Contrary to the results obtained under the low glucose concentration, it was observed that the fermentation properties of the recombinant and control strains were not quite different at 30°C. In both cases, most of glucose was consumed in about 24 h producing about 40 g/L ethanol with a yield of 40%. In the cultures at 40°C, glucose uptake and cell growth rates were retarded for both strains. Glucose was depleted at 33 h, which is significantly longer than time of glucose depletion at 30°C. The cell and ethanol concentrations started to decrease at 30 h, even before the depletion of glucose. At this temperature, the maximum cell concentration of the recombinant strain was 5% higher than that of the control showing the improved heat tolerance of the recombinant strain. While the cell growth of the strains was severely decreased by about 50% according to the temperature rise, the ethanol production was less affected by 4 ~ 9% by such heat stress. The ethanol yield at 40°C of the recombinant strain was 12% higher than that of the control strains. Moreover, the ethanol yield of the recombinant strain at 40°C was 43.7%, which was about 10% higher than that at 30°C.

The recombinant strain containing *salC* showed only slightly improved performances in flask cultures with 20 g/L of glucose at elevated temperatures of 40 and 42°C, similar to the results of stress sensitivity and viability tests. However, when the initial glucose concentration was increased up to 100 g/L, more meaningful results could be obtained in terms of ethanol production, especially. Under such condition with a high concentration of ethanol produced, the expression of *salC* played a great role of protecting cells from heat stress. Ethanol production at high temperature has been considered to be an important subject because maintaining the optimum fermentation temperature requires high cooling costs. A reduction in the costs associated with cooling is the first potential advantage of a high-temperature fermentation process [26]. There have been many reports described about the studies on the isolation or improvement of yeast strains to produce more ethanol at higher temperatures. Kiransree *et al*. reported thermotolerant strains of *S. cerevisiae* isolated could increase ethanol yield at 40°C as much as 40% [27]. Balakumar *et al*. reported ethanol yield and overall ethanol productivity of a thermotolerant strain of *S. cerevisiae* at 40°C were about 38% and 0.8 g EtOH/L/h, respectively [28]. In this study, the recombinant strain showed about 1.2-fold higher yield and 1.6-fold higher productivity than in the case of Balakumar *et al.*

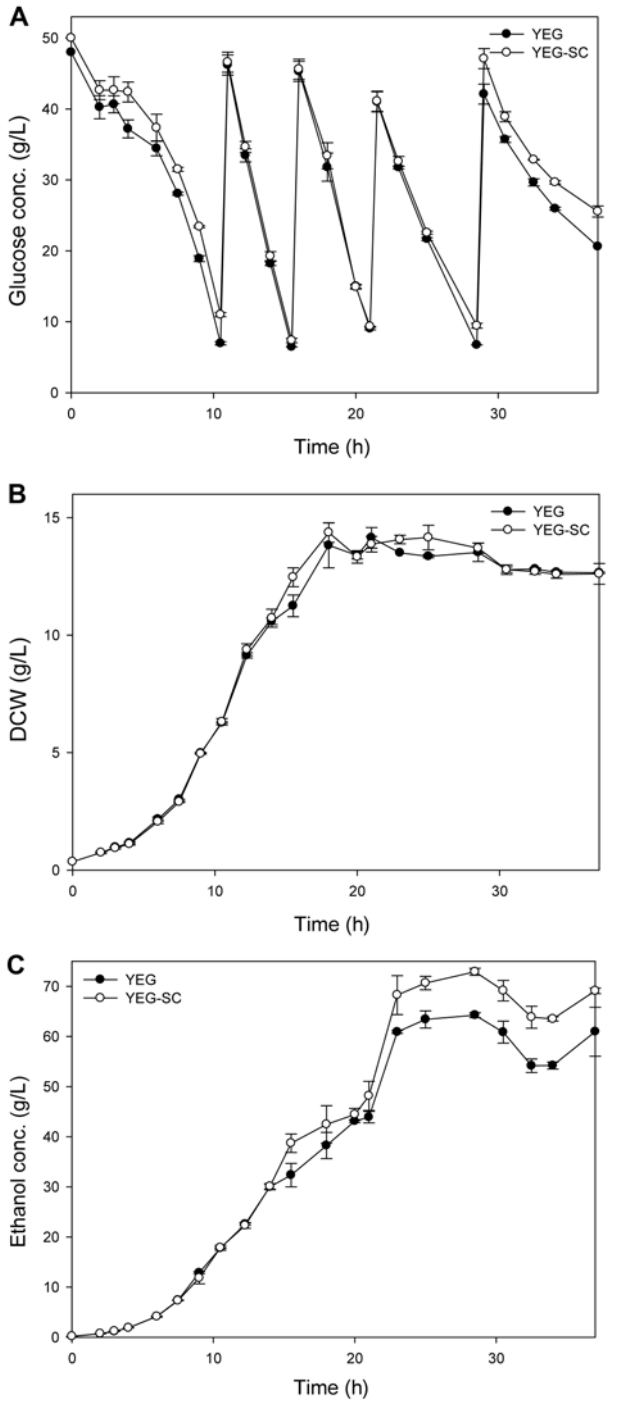

Fig. 5. Fed-batch fermentation of YEG-SC (open) and the control (filled) strain at 30°C. (**A**) Glucose uptake, (**B**) growth, and (**C**) ethanol production.

3.6. Fed-batch fermentation in bioreactor

In this experiment, fed-batch fermentation was performed to achieve a high ethanol concentration at the end. The performances of both strains were compared to each other at this high-ethanol level condition which was similar to an industrial practice for ethanol production.

We compared cell glucose and ethanol concentrations of the control and recombinant strain at 30°C (Fig. 5 and Table 5). The recombinant and control strains showed similar glucose uptake and growth behavior, so the feeding profiles of both strains were not significantly different. The ethanol concentration profiles of the recombinant and control strains began to show differences after 15 h, when the ethanol concentration was over 30 g/L. The ethanol concentration and the glucose consumption of both two strains began to decrease after 28.5 h. The broth volumes at 37 h when the culture was stopped were 2.4 L for both strains, and the ethanol concentrations were 69.1 and 61.0 g/L for the recombinant and control strains, respectively. In overall, the recombinant strain showed about 16% higher ethanol yield and about 13% higher ethanol productivity than the control.

Increasing levels of ethanol during a *S. cerevisiae* culture can severely inhibit cell growth and ethanol production in the later part of the culture. In particular, in the case of industrial yeast fermentation to produce ethanol to a high concentration, the most important chemical stress is ethanol toxicity, and the ability or inability of the yeast to tolerate the toxic effects of ethanol is of profound commercial significance [29,30]. Higher ethanol tolerance leads to smaller and cheaper equipment for ethanol production. The higher concentrations reduce the amount of water that must be removed in a final distillation step, thereby saving energy [31].

4. Conclusion

In recent years, it has been of great interest to search for a stress-tolerant yeast strain that is capable of producing substantial amounts of ethanol under various stress conditions in order to make the bioethanol process more productive [32]. Classical strain improvement methods have been successful in obtaining several industrial strains, but it is time-consuming and laborious for many repeated

Table 5. Key physiological data evaluated in the fed-batch fermentation of YEG-SC and the control strain at 30°C

Strain	Max. cell conc. (g/L)	Cell mass yield (g [dry wt]/g Glc)	Max. EtOH conc. (g/L)	EtOH yield (g EtOH/g Glc)	Overall ethanol productivity (g EtOH/L·h)
YEG	14.14 ± 0.44 (21 h)	0.12 ± 0.01	64.25 ± 0.50 (28.5 h)	0.42	2.25
YEG-SC	14.37 ± 0.42 (18 h)	0.16 ± 0.00	72.90 ± 0.71 (28.5 h)	0.49	2.56

rounds of random mutation and selection methods [33]. In this study, a putative trehalose biosynthetic gene of *salC* originated from *Streptomyces albus* was proven, functionally, to encode trehalose-6-phosphate synthase (TPS), and we observed that stress tolerance and ethanol production were enhanced by the introduction of *salC* gene into *S. cerevisiae* as well. Although many studies have been carried out on the relationship between stress tolerance and the metabolism of trehalose [5,6,8,9,13,14,34], only a few reports have been published on heterologous expression of trehalose biosynthetic genes in yeast [35,36]. Their main goals were to demonstrate whether putative genes encoded true trehalose biosynthetic enzymes and alter the functions of trehalose biosynthetic genes in yeast. The main goal of our research, however, was to develop stress tolerant strains and thus, to enhance ethanol productivity at a high temperature and/or a high ethanol concentration.

To our knowledge, this report describes the first application of a trehalose biosynthetic gene of different origin to develop high-stress tolerance yeast strains. We showed that heat and ethanol tolerances of yeast can significantly be improved by the expression of a trehalose-phosphate synthase-encoding gene originated from *Streptomyces*, which could be a very effective way to enhance ethanol productivity enhancement and thus, reduce the costs of bioethanol production.

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