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Increased production of S-adenosyl-L-methionine using recombinant *Saccharomyces cerevisiae* sake K6

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Abstract−*S. cerevisiae* sake K6 was the firstly isolated industrial strain to overproduce S-adenosyl-L-methionine (SAM). Although the strain has advantages over other strains, such as GRAS (generally recognized as safe) property, the *S. cerevisiae* K6 has not been further developed with DNA recombinant technology due to the lack of a proper genetic marker. In this study, UV mutagenesis was conducted with *S. cerevisiae* sake K6. With the method, a mutant of sake yeast with leucine auxotroph, K6-1, was isolated. The mutant showed comparable growth rate and SAM productivity with its wild type. Using the auxotroph as a genetic marker, a SAM synthase in *S. cerevisiae*, *SAM2*, was overexpressed in the mutant strain. This recombinant DNA technology successfully increased SAM productivity in sake yeast.

Key words: S-Adenosyl-L-methionine, *Saccharomyces cerevisiae* Sake, Auxotrophic Mutant, Recombinant DNA

INTRODUCTION

S-adenosyl-L-methionine (SAM), also known as SAM-e and AdoMet, is an essential metabolite in all living organisms. Its major function is methyl donation to nucleic acids, proteins, polysaccharides, lipids, cholesterols, etc. It is also a precursor to produce glutathione, an intracellular antioxidant, or polyamines [1,2]. SAM has been proven as a chemotherapeutic reagent with various clinical effects. It is well acknowledged to lessen alcoholic liver injury [3]. Also, the daily intake of SAM has been shown to reduce the symptoms of osteoarthritis [4], depression [5], and Alzheimer disease [6].

It was reported that SAM was produced in abnormally high level in sake yeast, *Saccharomyces cerevisiae* kyokai yeast [7,8]. *S. cerevisiae* sake K6 was the first strain to be developed to overproduce SAM. By supplementing methionine in the media, SAM was accumulated up to 28% of dry cell weight inside the cell [7]. Then, a few attempts were made to increase the SAM productivity in *S. cerevisiae* using ethionine- or nystantin-resistant mutants [9,10].

SAM is synthesized by SAM synthase, which makes a conjugation reaction between methionine and adenosine triphosphate (ATP). Recently, recombinant *Pichia pastoris* that overexpresses SAM synthase has become a popular host to overproduce SAM [11]. After then, several DNA recombinant methods have been applied to *P. pastoris*, including knocking out cystathionine-β synthase [12], expressing bacterial hemoglobin [13], and adding oxygen vector [14].

Meanwhile, DNA recombinant technology has not been applied to *S. cerevisiae* sake yeast, which is the first industrial strain to produce SAM. The reason might be the lack of a proper genetic marker in sake yeast. In this study, we isolated a leucine auxotrophic mutant of *S. cerevisiae* sake K6. Using the auxotroph as a marker, a DNA recombination was conducted in the sake yeast for overproduction of SAM.

EXPERIMENTAL SECTION

1. Strains and Media

S. cerevisiae K6 (Sake yeast kyokai No. 6) was acquired from KCTC (Daejeon, Korea). *E. coli* strain DH5α was used for plasmid construction. LB medium (sodium chloride 1%, tryptone 1%, yeast extract 0.5%, ampicillin 50 μg/ml) was used for *E. coli* selection and YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for *S. cerevisiae*. Whenever necessary, 0.5% methionine, 0.5% glycine, and/or 0.02% galactose were added for SAM production. For screening auxotrophic mutants, SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids) as well as Leu^-, His^-, Ura^-, and Ade^- medium (SD medium with 0.069% (w/v) CSM-LEU, CSM-HIS, CSM-URA, and CSM-ADE, respectively) were used. For making a solid medium, 2% agar was added to the media. Flask cultures were performed with 20 ml media at 30 °C, 250 rpm in 250 ml baffled flask (PYREX®, Seoul, Korea). Optical density was measured at 660 nm by using UV-mini1240 (Shimadzu, Kyoto, Japan).

D-glucose and agar powder were purchased from Dae-Jung Chemicals Ltd (Siheung, Korea). Yeast extract, peptone, yeast nitrogen base, and tryptone were purchased from Becton, Dickinson and company (Franklin Lakes, NJ, USA). Sodium chloride, amphicillin, galactose, mehionine, and glycine were purchased from Sigma Aldrich (St Louis, MO, USA). CSM-LEU (complete supplement mixture minus leucine), CSM-HIS, CSM-URA, and CSM-ADE were purchased from MP Biomedicals (Solon, OH, USA).

2. UV-mutagenesis

S. cerevisiae K6 was grown in a test tube to 10^8 CFU/ml for 24 h at 28 °C on YPD medium. Then, the cells were diluted 10^5 fold and were spread on YPD agar plates. The plates were placed under 254

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‡This article is dedicated to Professor Chul Soo Lee in commemoration of his retirement from Department of Chemical and Biological Engineering of Korea University.

nm UV lamp at a distance of 25 cm for 1 min. Then, the plates were incubated at 30 ºC for 48 h. The developed colonies were placed on the replica plate with SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids) agar. The colony that showed clear growth deficiency in the SD medium was further tested in four different media: Leu^-, His^-, Ura^-, and Ade^- media (SD medium with CSM-LEU, CSM-HIS, CSM-URA, and CSM-ADE 0.069%, respectively) to specify the auxotroph. The isolated leucine auxotrophic mutant was named as *S. cerevisiae* K6-1.

3. Cloning of *SAM2* Gene

To construct the expression vector for *SAM2* genes, pESC-Leu was purchased from Stratagene (La Jolla, CA, USA). A set of primers, 5'-GGATCCATGTCCAAGAGCAAAACT-3' and 5'-AAGCTTGCATAAAGAAAGGGATTG-3', was used for PCR amplification of *SAM2* gene with *S. cerevisiae* Y2805 chromosome as a template. The amplified gene was cloned into T-easy vector (Promega, Madison, WI, USA) and then cloned under *GAL1* promoter of pESC-Leu using BanHI and HindIII restriction enzymes (Promega). The resulting vector, pESC-Leu-*SAM2*, was transformed into *S. cerevisiae* K6-1 by lithium acetate method and selected on the Leu^- medium. *S. cerevisiae* K6-1 containing pESC-Leu-*SAM2* was named as SK6-1.

4. Real Time RT-PCR

To validate the overexpression of the *SAM2* gene, real time RT-PCR was conducted. The *S. cerevisiae* SK6-1 strain was grown in Leu^- medium for 48 h with 0.02% galactose. The yeasts were harvested by centrifugation at 2,500 rpm for 5 min. Total RNA was purified with RNeasy purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The quality of purified RNA was examined in agarose gel electrophoresis. cDNA was synthesized using 500 ng total RNA with iScript™ cDNA synthesis kit (BIO-RAD, Hercules, CA, USA) and used as a template for real time PCR.

The primers used for real time PCR were 5'-ATGACGATTCTGCCAAGGGTTTC-3' for sense and 5'-CCAGCACCTAAGTCTTCTAAGC-3' for antisense strand of *SAM2* gene and 5'-TCCCAGGTATTGCCGAAAGAATG-3' for sense and 5'-TGTTGGAAGGTAGTCAAAGAAGCC-3' for antisense strand of actin gene (*Act1*). These primers were mixed with 50 ng/ml of cDNA and iQ™SYBR Green Supermix (BIO-RAD). PCR was conducted in MiniOpticon (BIO-RAD) with 40 cycles of 20 sec at 94 ºC, 20 sec at 56 ºC, and 20 sec at 72 ºC. The PCR curves were imported in iCycler iQ program (BIO-RAD), and the expression level was calculated with comparative crossing threshold (Ct) method.

5. Extraction of SAM and HPLC Analysis

For measuring intracellular concentration of SAM, yeast pellets were suspended in 1.5 M perchloric acid and rotated for 2 h on ice. After centrifugation, the pellets were removed. Using the supernatant, SAM was quantified with HPLC ACME-9000 (Young-lin instrument, Anyang, Korea) with C18 column (BIO-RAD) by using a UV lamp at 254 nm and 0.5 M ammonium formate (pH 4) as a mobile phase at 1 ml/min velocity. The quantity of SAM was calculated from the peak area based on a standard calibration curve. SAM standard was purchased from Sigma Aldrich.

RESULTS AND DISCUSSION

1. Construction of *S. cerevisiae* K6-1

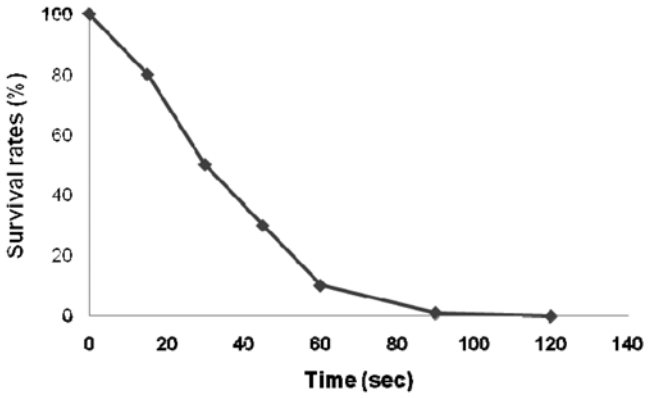

Fig. 1. The survival rate of *S. cerevisiae* sake K6 under 254 nm UV lamp on the agar plate. The results are averages of three replicated experiments.

For applying DNA recombinant technology in yeast, auxotroph is usually used as a selection marker. Although the industrial strain *S. cerevisiae* K6 was screened from the production site of a Japanese alcoholic beverage and confirmed as an extraordinarily high producer of SAM, it did not have any auxotrophic selection marker. Without a selection marker, further improvement of the strain using DNA recombinant technology was not possible. Therefore, we conducted a UV-mutagenesis experiment to isolate auxotrophic mutant of sake yeast K6 as described in the experimental section. To optimize the UV-mutagenesis condition, a survival rate of K6 under 254 nm UV lamp was examined (Fig. 1). Based on the rate, the treatment for 60 sec with 10% survival rate was chosen as an experimental condition.

S. cerevisiae K6 was diluted into 10^3 CFU/ml and spread on the YPD agar plate. After UV treatment, total of 3000 colonies were picked and grown in both YPD and SD media. Among those, two colonies were grown slowly and one was not on SD medium. To clearly identify the auxotroph, those colonies were re-examined on Leu^-, His^-, Ura^-, and Ade^- media. The former two colonies did not show any growth deficiency in all four media, while the latter one was unable to grow on Leu^- medium (Fig. 2). We successfully isolated a leucine auxotrophic mutant of *S. cerevisiae* K6 and named it as K6-1.

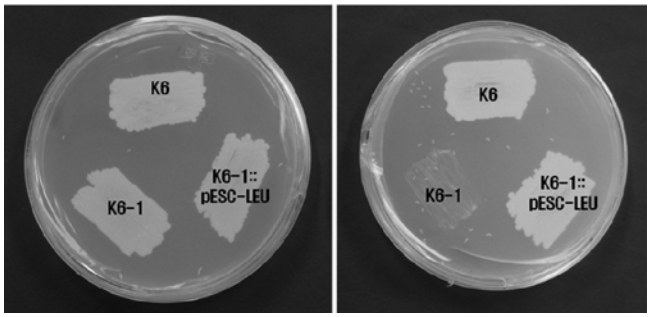

Fig. 2. The auxotrophic phenotype of the isolated auxotrophic mutant K6-1. The left plate was the strains grown in YPD medium and the right one in Leu^- medium. The growth defect of K6-1 in Leu^- medium was rescued by transformation of pESC-LEU plasmid.

To characterize the exact genetic marker of this strain, pESC-LEU was transformed into the strain. The transformation rescued the growth deficiency of K6-1 on Leu⁻ medium (Fig. 2), meaning that *LEU2* gene can be used as a selection marker for DNA recombination in this strain.

2. Overexpression of *Sam2* Gene in Recombinant *S. cerevisiae* K6-1

It has been known for a while that *S. cerevisiae* has two SAM synthase genes. Between the two, *SAM1* underwent strong repression by addition of methionine in the media, while *SAM2* was induced in the same condition [15]. Since methionine is a precursor for SAM synthesis, overexpression of *SAM2* with methionine feeding could result in SAM overproduction. *S. cerevisiae SAM2* gene was already utilized for overproducing SAM in *P. pastoris* [11]. To overexpress this gene in *S. cerevisiae* K6-1, the gene was amplified from *S. cerevisiae* chromosome by PCR and cloned in pESC-LEU under *GAL1* promoter (Fig. 3a). The resulting plasmid was transformed into *S. cerevisiae* K6-1 and colonies were selected on Leu⁻ medium. One of the colonies was taken and named as SK6-1.

To verify the overexpression of *SAM2* gene in SK6-1, the mRNA level of the gene was quantified with real time RT-PCR. Three strains, K6, K6-1, and SK6-1, were grown in YPD medium for 48 h and 0.02% (w/v) galactose was added to the medium at 8 h after inoculation for induction of *SAM2* gene. The RNAs were purified and reverse transcribed into cDNAs. Real time PCR was conducted for *SAM2* and *ACT1* genes as described in the experimental section (Fig. 3b). Based on the comparative crossing threshold (Ct) value with the housekeeping gene, *ACT1*, as a reference, the expression level of *SAM2* gene was computed as 30-fold increase in SK6-1 compared to K6 or K6-1. The real time RT-PCR data proved the success of *SAM2* overexpression in recombinant *S. cerevisiae* sake K6.

3. Productivity of SAM in the *S. cerevisiae* K6 and its Derivatives

Although *S. cerevisiae* K6-1 was successfully isolated, the random mutations might deteriorate the SAM productivity in the strain. The SAM productivity of *S. cerevisiae* K6-1 was compared with K6 in YPD medium supplemented with 0.5% methionine and 1% galactose (Fig. 4). Fortunately, *S. cerevisiae* K6-1 did not show any decrease in growth rate as well as SAM productivity compared to K6. The auxotrophic mutant had very similar phenotype with its wild type in terms of SAM production.

The recombinant strain SK6-1 with *SAM2* overexpression in K6-1 was grown in the same condition. As expected, SAM productiv-

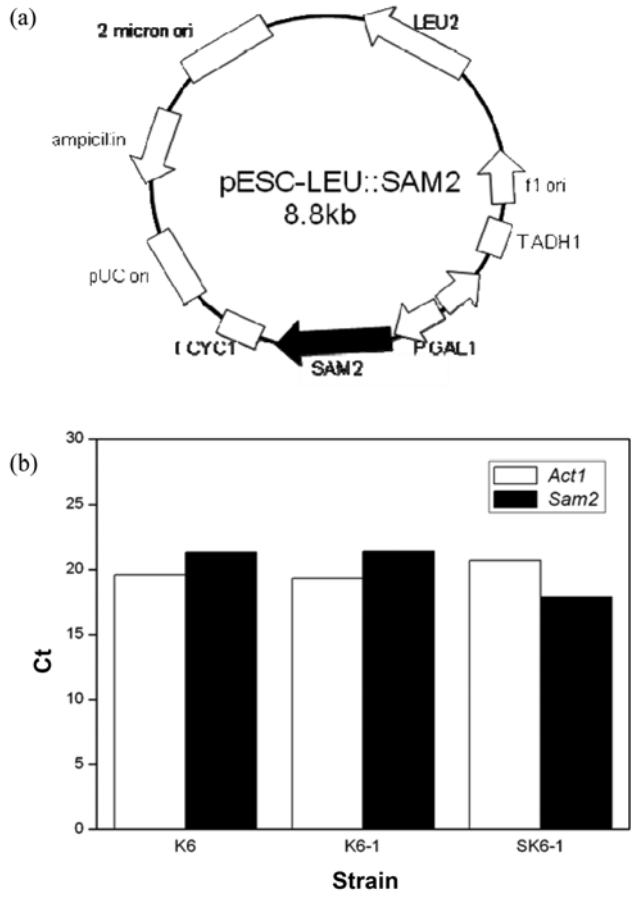

Fig. 3. The map of a multi-copy plasmid having *SAM2* gene under *GAL1* promoter (a) and the verification of its overexpression by real time RT-PCR after induction with 0.02% galactose (b). The lower Ct (crossing threshold) is, the higher the gene expression level. The gene expression level of *ACT1* was used as a reference.

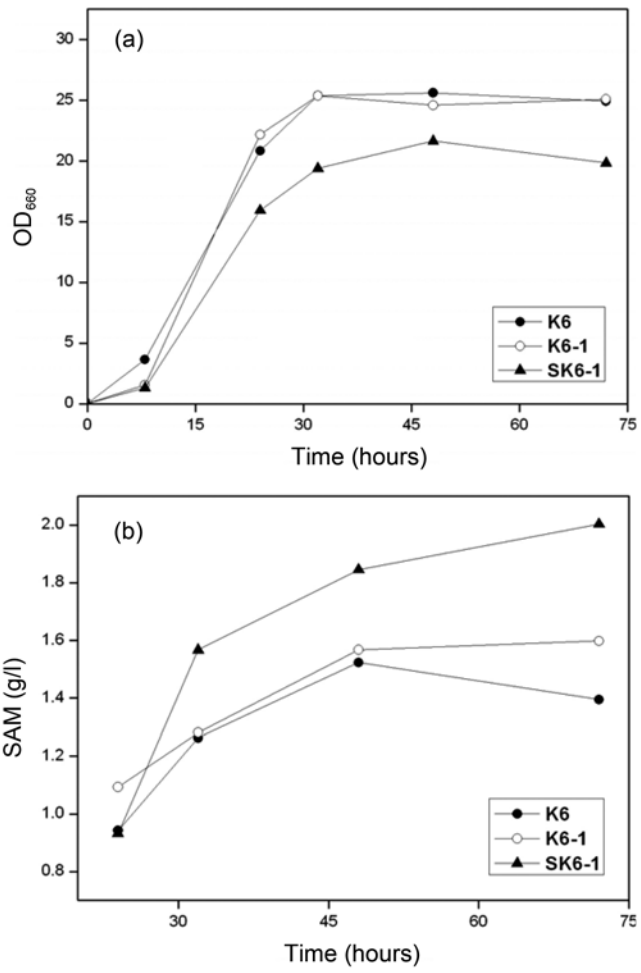

Fig. 4. Cell growth (a) and SAM productivity (b) of K6, K6-1, and SK6-1 in YPD medium with 0.5% methionine and 1% galactose.

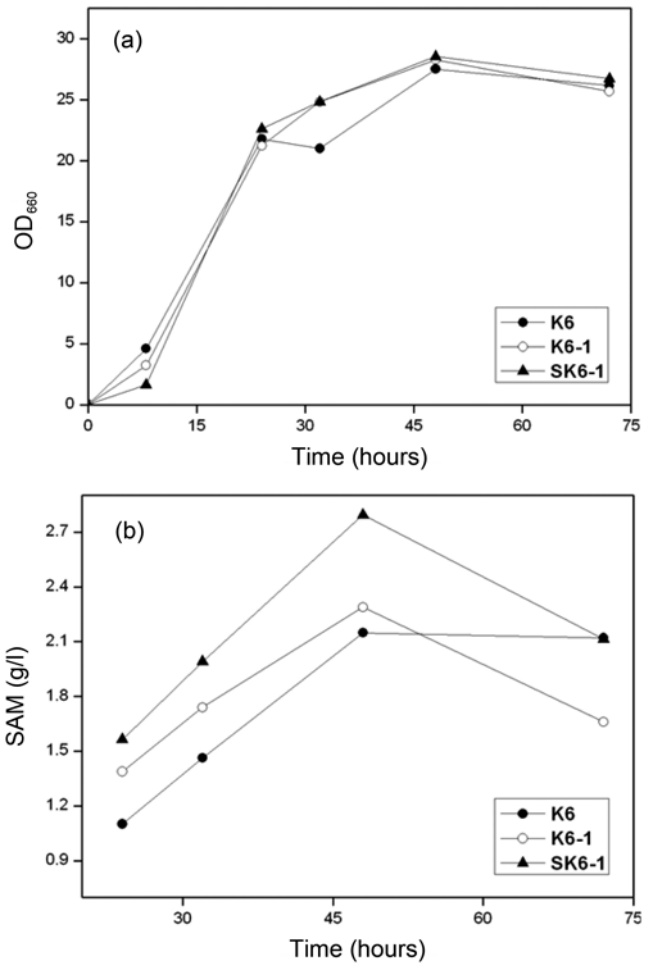

Fig. 5. Cell growth (a) and SAM productivity (b) of K6, K6-1, and SK6-1 in YPD medium with 0.5% methionine, 0.5% glycine, and 1% galactose.

ity of SK6-1 was greater than K6 and K6-1. Not only was the total productivity increased (Fig. 4b), but the specific SAM productivity in SK6-1 was increased to 0.35 g/g DCW compared with 0.22 g/g DCW of K6 and K6-1. However, SK6-1 did not grow as well as other strains in this condition, which resulted in reduction of total productivity (Fig. 4a).

It was suspected that the reduction of growth rate in SK6-1 was caused by limitation of methyl group in the cell. Serine, glycine, and formate are potential sources of the methyl group in the cell [16]. Therefore, 0.5% (w/v) serine, glycine, and formate were individually supplied to the medium. Interestingly, the growth rate of SK6-1 was completely recovered only by the addition of glycine. Three strains did not show any difference in the growth rate with supplement of glycine (Fig. 5a). SAM productivity was also greatly improved in all three strains. The maximum SAM productivity was increased from 2.0 g/*l* to 2.8 g/*l* in SK6-1, while from 1.6 g/*l* to 2.3 g/*l* in K6 and K6-1 by the flask culture (Fig. 5b). However, the specific SAM productivity in SK6-1 was not improved by the addition of glycine.

CONCLUSION

A very interesting phenotype of *S. cerevisiae* sake K6 was the accumulation of a large amount of SAM in the cell. After the K6 strain had been isolated, medium and culture condition optimization was conducted for increasing SAM productivity. However, a phenotype study or a strain development with DNA recombinant technology could not be applied to this strain due to the lack of a genetic marker. Therefore, we conducted UV mutagenesis to *S. cerevisiae* sake K6 to make a selection marker. Fortunately, a mutant with leucine auxotroph, K6-1, was successfully isolated from 3,000 tested colonies. This mutant did not show any decrease in growth rate and SAM productivity in flask culture. The leucine auxotroph was overcome by transforming *LEU2* gene. Using *LEU2* gene as a selection marker, a plasmid with *SAM2* under *GAL1* promoter could be introduced into K6-1. The recombination sake strain, SK6-1, showed improved SAM productivity, but decrease in growth rate. By supplying glycine in the medium, the growth rate of SK6-1 was recovered as much as K6 and K6-1 and 2.8 g/*l* SAM productivity was achieved with SK6-1.

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