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RAPID COMMUNICATION

Overexpression of ethionine resistance gene for maximized production of S-adenosylmethionine in *Saccharomyces cerevisiae sake* kyokai No. 6

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Abstract−In our previous work, we developed an auxotrophic mutant of *Saccharomyces cerevisiae sake* kyokai No. 6 strain for the selection of recombinant DNA. Sake yeast can accumulate high amount of S-adenosylmethionine (SAM) that is effective for treating depression, Alzheimer's disease and so on. In this work, we further introduced *SAM2* gene and *ERC1*, a frame-shift mutant of *YHR032w*, to maximize the production of S-adenosylmethionine in *S. cerevisiae sake* K6. The recombinant strain accumulated SAM about half of its dry cell weight.

Key words: S-adenosyl-L-methionine, *Saccharomyces cerevisiae sake*, Metabolic Engineering

INTRODUCTION

S-adenosylmethionine (SAM) is an essential metabolite in all living organisms and the second most abundant cofactor [1] because it donates its methyl group to various molecules including proteins, neurotransmitters, nucleic acids, phospholipids, etc. [2]. Moreover, SAM has the potential for various clinical treatments. Because of its anti-stress activity, SAM was reported as an effective therapeutic agent for treating liver disorder [3], depression [4], Alzheimer's disease [5] and so on.

Despite its usefulness, SAM has not received particular interest from metabolic engineers. Only few trials were made to overproduce SAM in yeast [6], because *Saccharomyces cerevisiae sake* kyokai No. 6 was known to highly accumulate SAM without any genetic modification. In our previous work, we isolated an auxotrophic mutant of this strain to introduce a recombinant DNA for metabolic engineering [7]. In this study, we further introduced *ERC1* into our previous auxotrophic strain to maximize SAM production, because the expression of *ERC1* was known to make strains more tolerant to ethionine and accumulate more intracellular SAM [8]; moreover, sake yeast actively expresses *ERC1* gene compared to wild type yeast [9]. In our result, strain ES1, which expresses both *SAM2* and *ERC1*, accumulated SAM about half of its dry cell weight in the intracellular region.

EXPERIMENTAL SECTION

1. Strains and Media

S. cerevisiae K6 (Sake yeast kyokai No. 6) was purchased from KCTC (Taejeon, Korea). LB medium with ampicillin (sodium chloride 1%, tryptone 1%, yeast extract 0.5%, ampicillin 50 μg/ml) was used for *E. coli* selection and YEPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for cultivating sake yeast. Flask culture was 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 using UV-mini1240 (Shimadzu, Kyoto, Japan).

2. Cloning of *SAM2* and *ERC1*

To construct the expression vector, pESC-*LEU* was purchased from Stratagene (La Jolla, CA, USA). Endogenous *SAM2* gene was cloned under *GAL1* promoter of pESC-*LEU* as previously described [6]. *ERC1* gene was amplified as described below and cloned under *GAL10* promoter of the pESC-*LEU* as well as pESC-*LEU*::*SAM2* using NotI and BglII. The vectors were constructed in *E. coli* DH5α. The resulting vectors were transformed into *S. cerevisiae* K6-1 strain [6] by lithium acetate method and selected on the Leu^- medium. The K6-1 strains containing pESC-*LEU*::*ERC1* and pESC-*LEU*::*SAM2*& *ERC1* were named as E1 and ES1, respectively.

3. Overlap Extension Polymerase Chain Reaction for Amplifying *ERC1*

Overlap extension PCR was exploited to introduce a deletion mutation into *YHR032w*. In the first round of PCR, the first fragment of *ERC1* was amplified by a set of primers, E1 (5′-GCGGCCGCATGTCTAAACAAT-3′) and E2 (5′-CCCACCTATTTTTTGTCTCCC-3′) and the second one was by E3 (5′-GACAAAAAATAGGTGGGTACA-3′) and E4 (5′-AGATCTTTTTATGATGTATGGGTC-3′). The underlined sequences in E1 and E4 primers indicate engineered restriction enzyme sites. Then, the PCR products of the first and second fragment of *ERC1* and *Taq* polymerase were mixed and denatured, annealed, and extended five times for 30 s, 30 s, and 1 min, respectively. Finally, a set of primers, E1 and E4, was added to the mixture and the second round of PCR was performed to amplify full-sized *ERC1* gene. The *ERC1* PCR product was cloned into T-easy vector (Promega, Madison, WI, USA) for further recombination.

4. Extraction of SAM and HPLC Analysis

The extraction and measurement of SAM referred to our previ-

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ous report [7]. Briefly, yeast pellets were suspended in 1.5 M perchloric acid and rotated for 2 h on ice. After centrifugation, the pellets were removed and the supernatants containing SAM were analyzed using HPLC. The quantity of SAM was calculated by peak area based on standard calibration curve. The SAM standard was purchased from Sigma Aldrich (St Louis, MO, USA).

RESULTS AND DISCUSSION

1. BLAST Search Verified that *ERC1* is Frame-shift Mutant of *YHR032w*

ERC1 was a frame-shift mutant of a putative protein and caused ethionine resistance and unusually high accumulation of SAM in *S. cerevisiae* [8]. Using protein BLAST search and sequence alignment tool, we found that *ERC1* was a frame-shift mutant of *YHR032w*. The product of the gene *YHR032w* is regarded as a putative substrate of the cAMP-dependent protein kinase (PKA) [10]. The PKA controls the transcription factor Opi1 which represses *SAM2* transcription via binding to UAS_{INO} in the upstream of *SAM2* coding region [11]. Therefore, we postulated that the product of the gene *YHR032w* is a downstream signaling protein of PKA signaling pathway and is related to the SAM metabolic pathway. Thus, the impairment of signal transduction in the regulation of SAM by overexpression of abnormal *YHR032w* (*ERC1*) could cause unusual hyperaccumulation of SAM.

2. Cloning of *ERC1* Gene and Introducing into Recombinant Sake Yeast K6-1

In the previous work, we developed a leucine auxotroph of *S. cerevisiae* sake, K6-1, and *SAM2* (encoding SAM synthase) was overexpressed in the strain by recombinant DNA technology. In that work, we achieved 2.0 g/L SAM concentration without glycine supplement. We further constructed the plasmid to overexpress *ERC1* (Fig. 1), which is known to enhance SAM accumulation [8] and examined the effect of *ERC1* overexpression in sake yeast. Deletion

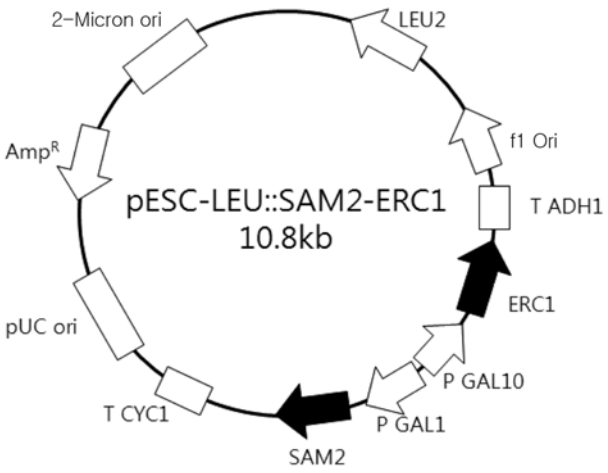

Fig. 1. The construction map of plasmid pESC-LEU::*SAM2*& *ERC1*. *SAM2* and *ERC1* were expressed in same plasmid under *GAL1* and *GAL10* promoters, respectively.

of one adenosine from 1628- to 1634-residue (5′-AAAAAAA-3′) of *YHR032w* makes a frame-shift mutation and ethionine resistant phenotype. Therefore, we applied overlap extension PCR [12] to introduce deletion mutation in *YHR032w* and to express the frame-shift mutant, *ERC1* (Fig. 2).

3. *ERC1* Overexpression Enhances S-adenosylmethionine Accumulation in Sake Yeast

In the *ERC1* overexpressed strain, E1, and both *SAM2* and *ERC1* overexpressed strain, ES1, showed slightly retarded growth (Fig. 3(a)). Compared to control strain K6-1, *ERC1* overexpressing strain E1 showed elevated SAM productivity. Without the supplement of glycine, we achieved above 2 g/L concentration of SAM in ES1 strain with flask cultivation (Fig. 3(b)). Moreover, we observed the accumulation of SAM reached to almost 50% of dry cell weight in

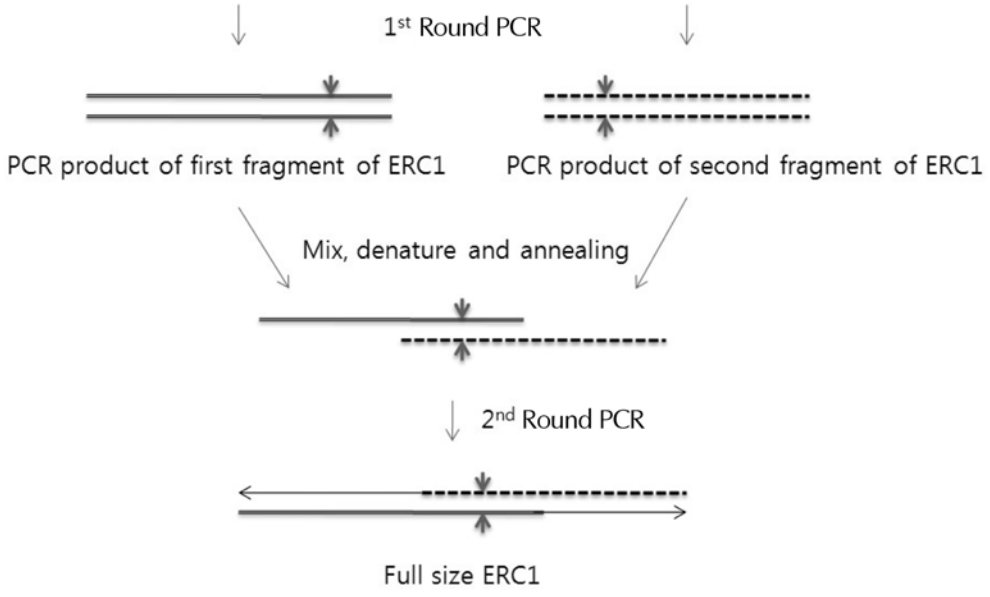

Fig. 2. Introduction of a deletion mutation in *YHR032w* using overlap extension PCR. Arrow indicates the introduced deletion mutation by primers.

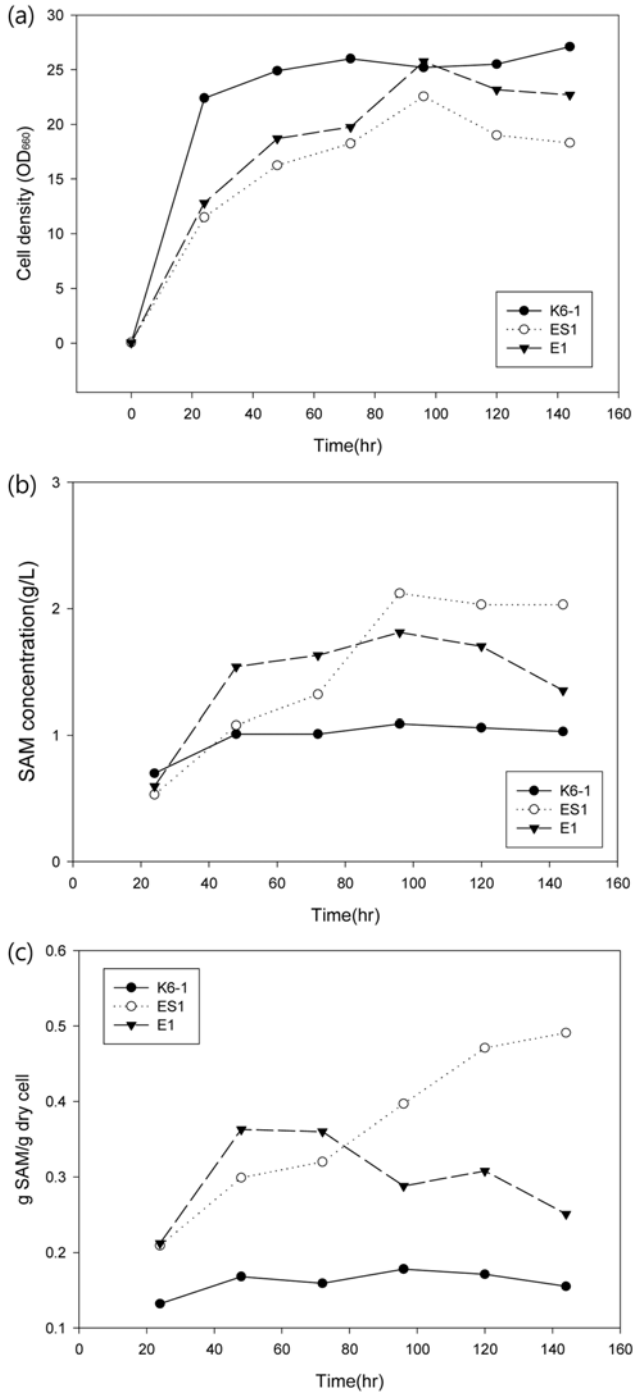

Fig. 3. The cell density (a), concentration of S-adenosylmethionine (b) and weight percentage of accumulated S-adenosylmethionine (c) of each strain.

the late-stationary phase in the ES1 strain (Fig. 3(c)). Because it is presumed that the half of cell mass is almost the maximum amount of metabolite storage in the cell [13], we concluded that this might be the maximized productivity of SAM in sake yeast.

CONCLUSION

Few trials were made to metabolically engineer *S. cerevisiae* for producing more SAM [14]. We engineered *S. cerevisiae sake* kyokai No. 6, which can already accumulate a high level of the SAM. In this work, we introduced *SAM2* (encodes SAM synthase) and *ERC1* (encodes a frame-shift mutant of *YHR032w*) into a leucine auxotroph *S. cerevisiae sake* kyokai No. 6 (named as K6-1). We observed that *SAM2* and *ERC1* introduced strain (ES1) stored remarkably high amount of the SAM. Because the SAM is accumulated in the vacuole in yeast [13], a half of dry cell mass could be almost the maximum capacity of metabolite storage in the cells.

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